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Current Topics in Malaria

Edited by Alfonso J. Rodriguez-Morales

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http://dx.doi.org/10.5772/61868
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Notice

First published in Croatia, 2016 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Current Topics in Malaria
Edited by Alfonso J. Rodriguez-Morales
p. cm.
Print ISBN 978-953-51-2789-5
Online ISBN 978-953-51-2790-1
eBook (PDF) ISBN 978-953-51-4144-0

Meet the editor

He received his Doctor of Medicine (MD) degree from the Universidad Central de Venezuela, Caracas, and he got his Master of Science (MSc) degree in Protozoology from the Universidad de Los Andes, Trujillo, Venezuela. He received his Diploma in Tropical Medicine & Hygiene (DTM&H) from the Universidad Peruana Cayetano Heredia, Lima, Peru, and University of Alabama at Birmingham, Birmingham, Alabama, USA. He is a fellow of the Royal Society for Tropical Medicine and Hygiene (FRSTMH), London, UK. He is a fellow of the Faculty of Travel Medicine (FFTM) of the Royal College of Physicians and Surgeons of Glasgow (RCPSG), Glasgow, Scotland, UK, and a fellow of the American College of Epidemiology (FACE), USA. He is also a candidate to Doctor in Parasitology (PhD) at the Universidad Central de Venezuela. Prof. Rodriguez-Morales is the president of the Travel Medicine Committee, Pan American ID Association. He is the secretary of the Colombian ID Association, a senior researcher of the Colciencias (2015–2019), and a professor in Universidad Tecnológica de Pereira, Pereira, Risaralda, Colombia (H index = 17).

Contents

Preface

The number of malaria cases globally fell from an estimated 262 million in 2000 (range: 205–316 million) to 214 million in 2015 (range: 149–303 million), a decline of 18%. Most cases in 2015 are estimated to have occurred in the World Health Organization (WHO) African Region (88%), followed by the WHO South-East Asia Region (10%) and the WHO Eastern Mediterranean Region (2%). The incidence of malaria, which takes into account population growth, is estimated to have decreased by 37% between 2000 and 2015. In total, 57 of 106 countries that had ongoing transmission in 2000 have reduced malaria incidence by >75%. A further 18 countries are estimated to have reduced malaria incidence by 50–75%. Thus, the target of Millennium Development Goal (MDG) 6 "to have halted and begun to reverse the incidence of malaria" (Target 6C) has been achieved (1). Besides that, malaria is still very important as a cause of morbidity and mortality in certain areas of some countries in the world. Even more, countries such as Venezuela have an opposite trend, in which malaria and other vector-borne diseases have significantly increased during the last decade (2). In addition, since 2014, severe forms of *Plasmodium vivax* have been recognized by the WHO (3-17). Still, the impact of malaria in pregnancy is important and is specially more recognized and studied in the Americas (12, 13). Therapeutical management and specially the development of antimalarial resistance are growing challenges for malaria control. Unfortunately, despite decades of research, still we do not have an effective licensed vaccine for malaria, but the goal is close and is anticipated to have it in the next following years.

With these conceptions in mind, this book includes different topics with regard to epidemiology, biology, clinical manifestations, treatment, and prevention, including entomological aspects, such as vaccines, of the wide spectrum of manifestations caused by *Plasmodium* spp. in humans and animals, trying to update the most significant research in many of them as well as to offer a multinational perspective on different aspects. This book has been organized in five major sections: (I) Clinical and Epidemiological Aspects, (II) Basic Science (III) Therapeutics and Antimalarials, (IV) Vaccines, and (V) Entomology and Vector Control. Section I includes topics related to history, epidemiology, seasonality, clinical presentations in pregnancy and children, as well as severe forms of disease for *P. falciparum* but also for *P. vivax*. Section II includes studies about avian malaria as well as the biology of gametocytes, highly important for blocking transmission vaccines, antimalarials, and control. Section III explores about new antimalarials as well as about the continuing threat of drug resistance. Section IV includes data and prospects about the vaccines for malaria. Finally, Section V discusses topics related to integral vector control, insecticides, and resistance and studies of secondary vectors, among other relevant aspects in the entomology and vector control of malaria.

Commissioning of this book by InTech has been related in part to my long commitment with malaria and other vector-borne and tropical diseases, since my undergraduate studies, working in infectious diseases, later as physician in the National Malaria Program and as rural physician for a PAHO project of surveillance of antimalarial drug resistance (AMI-

RAVREDA), which allowed me to work in endemic areas of Venezuela (Sucre), developing multiple studies, particularly those related to P. vivax severe malaria, which were recognized and cited in 2014 by the WHO (17), when accepted and published the Severe Malaria review including *P. vivax* malaria (2-5, 7-9, 11-13, 15, 18-22), as presented by me in a conference on that topic in 2014 in the International Congress of Parasitology in Mexico, including also our paper in Lancet about malaria in Venezuela (2). When I visited the Amazon jungle of Brazil in 2004 (Manaus and Coari, Amazonas), as visiting MSc student at Fiocruz with the direction of Dr. Claudio Tadeu Ribero, and couple years later Ifakara and Dar, Tanzania, Africa, in 2006, my interest for malaria also increased, and a year later, I received the Young Malaria Researcher of the Year—Malaria Awards Ceremony 2007 (Malaria Foundation International). In 2012, another national award was awarded to one of our studies, the Best Research Study of the Venezuelan Society for Infectious Diseases 2012 ("Comportamiento clínico y de laboratorio de malaria por *Plasmodium vivax*. Complejo Hospitalario Universitario Ruíz y Páez. Ciudad Bolívar, 2000–2010"). In addition, I had the opportunity to visit endemic areas of malaria in Peru, Iquitos, and Loreto as part of my Diploma in Tropical Medicine and Hygiene in 2009.

After moving in 2011 to Colombia, I have been involved in research of malaria in Risaralda, where we still keep working on this important tropical disease (5, 10, 14, 16, 22-25). Part of all this is a clear reflection of the work impulse at the Research Group Infection Public Health and Infection of the Faculty of Health Sciences of the Universidad Tecnológica de Pereira, directed by Dr. Guillermo Javier Lagos-Grisales. He is not just a partner, a colleague, and mainly a friend but also an extreme believer in our work in vector-borne and zoonotic diseases.

Following the same philosophy as we had on my four previous books with InTech, *Current Topics in Tropical Medicine* (26), *Current Topics in Public Health* (27), *Current Topics in Echinococcosis* (28), and *Current Topics in Chikungunya* (29), this book does not intend to be an exhaustive compilation, and this first edition has included not just multiple different topics but also a wide geographical participation from many countries of different regions of the world. Its online availability through the website of InTech, as well as the possibility to upload the complete book or its chapters in personal websites and institution repositories, allows it to reach a wide audience in the globe. Continuing on the series of Current Topics books, we are planning to develop in the future other projects such as *Current Topics in Giardiasis* (already in press), *Current Topics in Zika, Current Topics in Infectious Diseases*, and *Current Topics in Travel Medicine*, so if you are interested to participate in this endeavor as an author of one or more chapters, please contact us.

I would like to give a very special thanks to InTech (for the fourth time), particularly to Dajana Pemac (Publishing Process Manager), for the opportunity to edit this interesting and important book, as well as for their constant support.

I want to take the appropriate time and space, as I used to do, to dedicate this book to my beloved family (Aurora, Alfonso José, Alejandro, and Andrea, the neurologist) and particularly to my lovely wife, Diana. After 5 years of togetherness, I am so clear she is everything to me. She is the engine of my life. And due to her support, projects like this one would be not just possible, but successful. She is the highest blessing in my life, my soul mate, and my strongest support for any journey; she provides everything in my life, day to day. Also thanks to my friends and my undergraduate and postgraduate students of health sciences in Colombia, Venezuela, and around Latin America. Also it is time to say thanks to my collea-

gues at the Working Group on Zoonoses, International Society for Chemotherapy, and the Committee on Zoonoses and Haemorrhagic Fevers of the Colombian Infectious Diseases Society (ACIN). Also thanks to more than 45 members in 15 departments of the country of our Colombian Collaborative Network on Zika (RECOLZIKA) (www.RECOLZIKA.org), which I have the pleasure to chair, especially to my friends Wilmer E. Villamil-Gómez (author of this book), Carlos Franco-Paredes (author of this book and a partner in multiple malaria projects), Elia Sánchez and Melissa Arria (authors of multiple published studies with me), among many others across Colombia and Latin America. Also thanks to Dr. Juliana Buitrago-Jaramillo, the former Dean of the Faculty of Health Sciences, Universidad Tecnológica de Pereira, who supported most of the efforts of our research group, Public Health and Infection. Special thanks to my friend and colleague Dr. Guillermo J. Lagos-Grisales, MD, MPH. Also thanks to the current Dean, Dr. Rodolfo Cabrales, who has also been an enthusiast and a supporter of our research activity. Members of our research group and incubator consist of young and enthusiastic medical students, and some veterinary medical students as well as young medical doctors, who are pursuing significant improvements in the understanding of the epidemiology of zoonotic, vector-borne, parasitic, and in general infectious diseases, in our country with international projection, are duly acknowledged. Year 2016 has been highly productive for this recognized group, which is positioning as a leader in infectious disease epidemiology research in the coffee-triangle region and will position a leader very soon in the country.

Finally, I hope our readers enjoy this publication as much as I did reading the chapters of *Current Topics in Malaria.*

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Section 1

Clinical and Epidemiological Aspects

Chapter 1

Tertian Fevers in Catalonia in the Late Eighteenth Centuries: The Case of Barcelona (1783–1786)

A Methodological Proposal to Develop Studies over Endemic and Epidemic Malaria in Past Societies

Kevin Pometti

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64977

Abstract

In this chapter, we propose a broad perspective of the sources available for the development of studies of endemic and epidemic malaria in past societies. The complexity of malaria as a disease is related to a variety of elements (environment, climatic oscillations, and human production and cultivation patterns). Historically, the study of malaria was integrated into the study of fevers in general. Indeed, malaria is a protean disease that interacts in positive, negative, and synergetic ways with other eukaryotic, viral, and bacterial diseases. Because of that, the word "fevers" conflates a wide range of diseases and symptoms that can also help us to detect the prevalence of malaria and relationships between the disease and environmental factors. Terms such as fevers, intermittent fevers, agues, and marshland fevers can be easily found in historical sources, print sources, and a large amount of documentation produced by state-municipal authorities, by physicians, and found in burial records. In sum, these represent the diversity of points of view involved in our research. Using as an example the case of Barcelona in the late eighteenth century, we show some results based on a methodology with a strong interdisciplinary basis.

Keywords: malaria, environment, Spain, Barcelona, disease

1. Introduction

In the late eighteenth century, we find a general context in Catalonia in which institutions, sanitation, and society adapted to political changes marked by the application of the "Real

Decreto de Nueva Planta" [1], one of the most important consequences of the Spanish Succession War. This law was elaborated and applied in 1717 by Bourbon authorities to control traditional Catalan institutions, modify the pre-existing legislation, and also reshuffle the pre-existing structures to create a new assembly according to the interests of the new monarchy. At the same time, the "Principado" political model was introduced in Catalonia. This model was similar to that prevalent in the majority of European countries, in which the sovereign could formulate laws separately from the political community [2]. In this sense, throughout the eighteenth century, the Audience regent in conjunction with the Intendent and the General Captain were the authorities that presided over political decisions throughout the Catalan territories [3].

The city of Barcelona was not an exception concerning the application of the new laws. From December 1718 [4], the consequences were felt in urban structures, the city council, the society, and all sanitation institutions and sanitation professionals. The city fall on 11 September 1714 implied the suppression of the traditional city council (known as "Consell de Cent"), and the construction in 1715 of Ciudadela fortress. This fortification was built to keep the city population under military control (**Figure 1**).

Figure 1. MOULINIER. Plano de la ciudad y Puerto de Barcelona. 1806. ICGC, RM. 19425.

The construction of the Ciudadela fortress also changed the traditional morphological distribution of the city. In fact, 17% of the total urban area [5] was occupied by the fortress,

http://dx.doi.org/10.5772/64977

causing 6380 people to be displaced to the most crowded city area, the quarter of Sant Pere i Santa Caterina, and changing the traditional morphology of the "artisan house." The ancient structures were adapted to the necessities of accommodating the displaced population, "There are not so many houses in the city constructed in the present century that don't have three or four rooms, or flats, more of them four, and some even five flats. The referred houses are actually taller than they were in the past. Their interior structures owe more to economics than to health. It is common to form a flat with the essential rooms, including a kitchen, saloon and bed, and a little room. This is seen in places that were commonly used as one room, which now present too many subdivisions. The majority of these rooms have a water well very close to the latrines" [6].

In 1753, a new quarter, designed by the military engineer Jorge Próspero de Verboom, was constructed to take in the population surplus of a crowded city center in which people's quotidian life was quite marked by coexistence with textile industries, stagnant water channels (Rec Comtal), and the unhealthy sanitary conditions of commercial activities. Just 4.2% of the citizens moved to the new Barceloneta. This fact stunned travelers and visitors such as Arthur Young in 1787. "A quarter, called Barceloneta, it is entirely new and regular; their streets cross at right angles. Now: because of the absence of sailors, small businesses and artisans, the houses are low and small. One of the sides faces the docks. The streets are brightened up, but due to the high amount of dust, by a special attention to the wider streets, I cannot say that all of them are provided with flagstone pavement" [7].

The stagnant agriculture patterns of Catalonia in 1750–1760 pushed people to transfer to cities: cities that offered the possibility of importing wheat and export the surplus wine production to maintain the prices [8]. Between 1717 and 1787, the city population increased from 35,928 to 100,160 inhabitants, a 180% enlargement that translated into a population of 114,100 by the end of the century [9]. In consequence, cities like Barcelona saw a considerable increase in and a conflict with infrastructure not adapted to the process of intensive agriculture based on irrigation. Agricultural intensification and specialization had their negative effects in directly favoring the proliferation of unhealthy areas "*We face, as Young noticed, in one of the most advanced agriculture of Europe a dark side: the growth of unhealthy areas with delta fevers as a consequence of irrigation patterns*" [10].

At the end of the century, we find a city adapted to the Bourbon reforms and urbanized with new spaces concerning the construction of Ciudadela and the Ramblas development. The city council from 1784 managed the regulation of the new spaces in which textile industries could be placed—outside the city walls—as we see in the message contained in the Royal Chord of 22 May 1784 "*in attention to the multiplicity of cotton and wool factories that have been built inside this city in recent years, which is currently excessive, and is starting to affect the everyday lives of the citizens and residents of this city*" [11].

The urban reshuffle such as the construction of a new sewage system and the extension of hydraulic infrastructure was considerably restricted due to the reduction of the annual city council budget to a quarter of its previous value [12]. One of the worst consequences was felt in the quality of water, which for the most part was contaminated due to bad insulation conditions of latrines and cesspits. This issue was a common preoccupation for the physicians

of the city due to the constant obstruction of an outdated sewer system "*But we have to be very sorry that those sewers have not been continued with the spirit of the first builders. Because of the limited capacity that those sewers currently present, in most streets they cause the presence of stagnant areas in which we often see accumulated a high variety of material and water. In consequence the pollution builds up and all along the vents and particularly the sewers emerge occasionally the putrid vapours that fill the houses and streets with stinking air; and other times the same material overflows, causing an insupportable stench*" [13].

The progressive intensification of the manufacturing activity meant that by 1806 Barcelona took in more than 104 industries with a total of 12,000 workers [14]. These industries crowded Barcelona in the late eighteenth century, particularly in the "prados de indianas" [15] that were placed around the most important rivers. Especially near the Besós [16] River, where in 1784 alone, those industries employed an additional 8638 people [17].

1.1. The pursuit of a medical academy in Barcelona

Sanitation institutions are one of the most important aspects on which we will focus our attention because of the importance of physicians as the principal observers of weather, environment, and diseases of past societies, more importantly in the late eighteenth century. Indeed, sanitation professionals had a strong social position, high importance, and an ancient tradition concerning the application of epidemic prevention policies in Barcelona during the sixteenth and seventeenth centuries [18]. Since the latter half of the sixteenth century, the prestige acquired by the Estudi General—the medical school of Barcelona—signified the inclusion in the citizen oligarchy of the physicians attached to the Col legi de Doctor en Medicina de Barcelona, the professors of Estudi General, and the ancient Hospital of Santa Creu.

In a more general perspective, the Real Tribunal del Protomedicato [19], founded on 30 March 1477, prevailed as the central state institution to control public health, to regulate medical practice, and to verify the quality of drugs and remedies dispensed by the apothecaries. One of their institutional competences was to collect taxes from exam fees and fines to administer them and the investment of the funds thus obtained. In fact, since the sixteenth century, in the Crown of Aragon, those exposed competences were administered by the traditional institutional structures. Furthermore, an important objective of physicians from Barcelona was to obtain the title of Royal Protomedico.

This institutional position allowed physicians to profit from an important social protection and to maintain the public presence of a medicinal profession open to the arrival of new methods and ideas for the renewal of medicine. The arrival of the new scientific medicine, based on the resurgence of Hippocratism, was the starting point of a change in the way in which epidemics in the latter third of the seventeenth century were confronted and understood [20]. Physicians started to displace the theoretical teaching of medicine to incorporate Hippocrates' aphorisms. From then on, the medicine became an effort to understand nature, naturae conamen, and to expel from patients' bodies all infective material. Physicians had to note carefully all the symptomatology, symptomatum concatenatio, to increase the knowledge of the diversity and variety of illnesses that medicine must face [21]. Physicians Herman Boherhaave or Thomas

http://dx.doi.org/10.5772/64977

Sydenham emphasized the construction of a medical knowledge based on an historical perspective of illnesses. The context of scientific exchanges led to a progressive evolution of medicine to hygienists' policies of the late eighteenth century. These, however, had to face new problems derived from the confluence of supporters and detractors of the new medical improvements, especially at the beginning of the eighteenth century [22].

The consequences of the application of Royal Decrees to sanitation professionals and medical institutions caused the regression of Catalan medicine. The most important effects of these Decrees were the suppression of institutions like the "Estudi General" and the creation of a new university in Cervera [23] (1714–1717). This university did not gain the support of those physicians associated with the former "Estudi General," due to its failure to provide any kind of improvement in medical studies. Debauchery and the loss of institutional control over medical practice drew to Barcelona a considerable number of unlicensed practitioners. This together with the loss of productivity in medical literature, the lack of proper control over medical studies, the decline of medical presence in municipal institutions, and the decline of the social importance of physicians were the principal impulses for physicians to try to recover control of medical practice. Their goal was to instate a medical practice in which professional schools of surgeons and apothecaries prevailed, with the consequent continuous conflicts due to the overlap of institutional competences.

The Junta de Morbo of Barcelona—an institution dedicated to the prevention of epidemics—was also brought under the new institutional laws. In fact, the arrival of the Plague in Marseille in 1720 prompted a reshuffle of the epidemic prevention institutions in the central Superior Council of Sanitation of the Kingdom [24]. This institution, since its foundation on 28 August 1720 by the administration of Felipe V [25], had assumed the local functions of the pre-existing epidemic prevention bodies. In this sense, the diversity of the range of functions of those bodies was, in the case of Barcelona, extremely diversified. The jurisdiction of the Superior Council was considerable, covering Barcelona and the rest of Catalonia at the same time.

This broad purview is explained by the need to build an elaborate network based on the communication of the provincial territorial subdivisions that formed the Superior Council of Sanitation. In the case of Barcelona, the same Royal Audience that proposed city hall members acted as a communication medium between the Superior Council of Sanitation and the municipal city council [26].

In fact, as we can see in the documentation, the communication between physicians, surgeons, and municipal authorities was the principal point that helped to keep an effective prevention system in the city and to apply epidemic prevention laws and observe their effects throughout the territory. At a more local level, the drastic municipal budget reduction of the Bourbon City Council caused serious difficulties and limitations in relation to the executive capabilities of the institution.

The impact of the institutional changes discussed above revealed the importance of the historical trust in physicians and in university medicine as a legitimation of the collective of medical professionals who took part in the public health system. This was one of the

objectives of a new generation of physicians, primarily trained at Montpellier University, who arrived in Catalonia in 1740. In the program of the Academia Médico-Práctica de Barcelona, these physicians sought State support and the recognition of the utility of medicine [20].

Philip V's death in 1749 signified a more opportune time to request the reestablishment of University of Barcelona with the consequent opposite interests of the recently founded University of Cervera and the interest of Real Protomedicato. Barcelona in 1760 and 1770 was subject to an institutional impulse that crystallized in the origins of two scientific academies, the Academia Médico Práctica and the Academia de Ciencias y Artes [27]. The origins of both academies were immersed in a context in which European academies were under Royal or manorial protection to develop studies linked to the political interests. The constant failures concerning the reestablishment of the Medical College in 1754, 1769, and 1770 together with the necessity of a medical corporation with teaching responsibilities forced the authorities to suppress those aspects that could present friction or an overlap of competences with the Real Protomedicato [20]. However, the existence of a medical hospital in Barcelona to observe diseases and to collect medical reports brought about the study of the anatomical effects of illnesses by means of dissections. This inclination was one of the pillars of the program of the Academia Médico-Práctica de Barcelona that we can see reflected in the inaugural speech of Dr. Jaume Bonells: "It is not enough to know the causes of illnesses without healing them; only the Government can remedy the origins of those diseases, and it is necessary that the magistrates and physicians work together" [28]. Moreover, Bonells refocused the interest in medical studies over the general interest of a society immersed in a constant development. Those interests included agriculture, cattle raising, and those "useful sciences" from which the economy could profit.

The program elaborated by Bonells is of principal interest for our research in that it concerns the need to observe the relationship between illnesses and the environment and climate [29]. This is in line with the studies proposed by Sydenham and Baglivi, in which a necessary point of observation was the precise moment at which epidemics originated and spread through the territory: "*With the collection of meteorological records of each town, we could have already the medical history of every time and region, and through this medium, Medicine would be in a degree of perfection in which today remains considerably remote*" [28].

This program of the Academia Médico-Práctica was formally proposed by Joan Esteve, lieutenant of the Protomedicato, and Pere Güell, first examiner of Protomedicato, in Catalonia on 29 April 1770. Their proposal obtained the approval of the Royal Audience on 2 July 1770 and was ratified in the first assembly of the Academia Médico-Práctica of Barcelona [30]. The most important recognition is seen in the Royal Decree of 21 September 1786, delivered by Charles III, in which the statutes of the Academia were approved and placed under Royal protection. This recognition gave the Academia the right to use the Royal printer to publish their statutes. Finally, in February 1797, the monarchy granted the chair in Medicine [31] to the Real Academia Médico-Practica de Barcelona; as a consequence, instruction in medicine was officially re-established in Barcelona.

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2. Malaria and environment

Malaria and its strong relation to environment becomes one of the most interesting points in relation to develop studies centered on epidemics and their impact over past societies. Paludism is a disease which kept a strong link with landscapes of the past, human productive facilities and agricultural patterns, climatic oscillations, temperature, rainfall, and wind direction [32]. In consequence, we find necessary to offer a general approach to his ethology and symptomatology but, especially, in the particular ways in which the illness interacts with another diseases.

In 2015, *World malaria report*, shows us a regressive impact of the illness in comparison with the estimated values given for the year 2000 (262 million cases of malaria globally—214 million in 2015—and 839,000 deaths—438,000 deaths in 2015) [33]. However, malaria is still present as a resistant illness to vector control measures (insecticides) and to antimalarial treatments (particularly *Plasmodium falciparum*). In fact, malaria is one of the most ancient illnesses known by the humanity with references in China on 2700 BC, Mesopotamia 2000 BC, and Egypt 1570 BC, and in Hindu texts from sixth century BC [34]. An illness whose symptomatology was well known and his relation with environment well specified, as an example, like shows us Hippocrates in 410 BC "*Passed the dog days the fevers became sweat, but behind him did not disappeared; the fevers came back again, with a moderated duration, difficult to attach and without giving to much thirst. In too much patients the fevers stopped in seven and nine days but in others after eleven days, fourteen, seventeen and twenty-two days*" [35].

The link between stagnant waters and malaria is a traditional causal relation present in medical records of the eighteenth century, which worried physicians and authorities by the same way. As an example, Francisco Cerdán said in their *Discursos physico-medicos, politico-morales que tratan ser toda calentura hectica contagiosa, essencia del universal contagio, y medios para precaverlo* (published in 1752) that "*Juan Maria Lancisi, Physician of Clemente XI, testified, that being Aquileya one of the most important cities of Italy… could not to tolerate too much epidemics, caused by the putrid exhalations that came from the stagnant waters*" [36]. In words of Mary Jane Dobson, "*These were the 'silent' fevers creeping from house to house, along the channels of contamination, but eventually revealing their impact on the seasonal, annual and secular graphs of mortality peaks*" [37].

In the case of Barcelona, before and after the epidemic of 1783–1786, we found many proclamations given by the authorities focused in the prevention of flood impact and in the control of marshlands and lagoons, especially, in the two principal rivers that surrounded Barcelona —Besós and Llobregat rivers. In this sense, we have selected three examples in which the first is the proclamation published by order of Jacinto Pazuengos y Zurbarán, Governor of Barcelona, on 8 April 1780 [38]. The principal message transmitted by the Governor was focused on the establishment of preventive hygienist policies to promote the systematic clean of fields, paths, river banks, and stream flows. Due to the constant floods, the necessity to keep river bank boats in good conditions was also mentioned, and building irrigation ditches, building new houses on Besós-Llobregat river banks, and building up embankments on marshlands were specifically prohibited.

On 16 May 1784, Manuel de Terán, General of the Royal Army in Catalonia, [39] published a new proclaim that reminded the authorities about the observations published in the past proclaim of 1780 because "*It is quite common, that the extraordinary rains, and other irregular and violent accidents cause notable injuries to the road paths* (…) *this happens due to the failure to follow some useful orders to prevent those situations*" [39]. The new and most important measure improved in the municipal proclaim concerns the prohibition to build new cane cultivation rafts. In spite of the efforts of the authorities, it will be problematic to follow the proposed hygienic policies.

On 31 March 1787, Manuel de Terán [40] again published another proclaim. An advice that this time will consist to improve a systematic planting of trees to keep water stream controlled on Besós and Llobregat rivers. However, as we can see through the testimony of Francisco de Zamora the planting of trees was not completely applied in 1789, "*The River banks of the referred rivers and torrents, creeks, streams that run over the center of Barcelona's area, generally, are not planted with trees. There are only few trees planted by the land proprietors of some parts of Besós and Llobregat river bank areas*" [41].

In fact, the relation with malaria and some agricultural patterns based in irrigation has been a common problem all along the sixteenth to eighteenth centuries as we can find in many bibliographical references or through the sources. Especially in the eighteenth and nineteenth centuries, malaria was associated with the rice cultivation [42–44]. This was due to the proliferation of artificial flooded areas that ensured the production of an alimentary resource that could offer a high quantity of food with reduced cost: "*In Europe, rice cultivation areas have been, since their implantation, responsible of the endemics of malaria and also their epidemic forms; although the authorities had tried to limit and avoid this kind of agricultural practice. However the restrictions have been frequently broken, in some cases totally disobeyed because of rice culture was a source of high incomes*" [42]. Unhealthy flooded areas all along peninsular Mediterranean basin that became famous over ages and countries as exemplifies the testimony of Pierre Pauly in his work published in 1874: "*The Mediterranean area of Spain it's all along his basin an important source of epidemics: Intermittent fever, the bilious remittent fever they are common from one side to another, more or less, naturally, by the pass of years; and where a unfortunate meteorological constitution (overcast, cloudy, frequently calm) conjuncts with the active local causes, and epidemic took place, finding a terrain prepared for its development*" [45].

Concerning the etiological aspects of the illness, there are more than 200 types of malaria [46], only four affects to humans: *Plasmodium Falciparum, P. Vivax, P. Ovale*, and *P. Malariae*. Malaria is a eukaryotic disease transmitted through the bite of an *Anopheles* mosquito that starts the infectious process in the human host. However, there are secondary infection forms especially in endemic areas which consist, for example, in mother-to-child transmission [47] causing new-born's weight loss, injuring his immunological system and being children the principal victims of a high range of diseases [46]. Malaria's infectious life cycle starts with an initial stage in which sporozoites flow through the blood circuit until they are installed in liver cells and complete the initial infectious cycle. In the liver cells, sporozoites reproduce themselves through asexual reproduction and through the *lysis*, process in which liberate new merozoites —more than 30,000 merozoites [48]. This is the fact that causes the progressive destruction of

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blood cells each 24 hours (*P. Falciparum*), 48 hours (*P. Vivax*), and 72 hours (*P. Malariae*) [46]. This process causes malaria's paroxysm in which the most severe fever episodes [49] causing the traditional symptomatology of a malaria infection (chill, fever, and sweat) are concentrated. Malarial fevers can be continuous (if there are no fluctuations higher than 0.5°C in 24 hours), remittent fevers (if the temperature keeps over 1°C or more in 24 hours) or intermittent (if the temperature comes back to normality one or two times in 24 hours) [50].

After that, sexual gametocytes circle along human host blood vessels until another mosquito bites. Inside mosquito's stomach, gametocytes will start the sexual reproduction generating Oocysts if the minimal ambient temperatures are higher than 17°C for *P. vivax* or up to 20°C to *P. falciparum*. This process closes the infectious life cycle when new sporozoites are released into the salivary glands of the mosquito ready to start another asexual reproduction cycle in the human host.

One of the most important consequences of malaria is that it causes the progressive destruction of human blood cells, generating a state of anemia. However, the most common symptomatology of the disease can be similar to less aggressive virus diseases causing abdominal pain, diarrhea, discomfort, fatigue, fever, headache and respiratory disorders in patients. There are also some physical and most appreciable symptoms such as jaundice and splenomegaly. In which the last one appears in adults after a series of malarial relapses but it is faster and easier to detect in kids.

However, the most interesting aspect is the immune system suppression that signifies a high risk of coinfection. A question that should be studied in detail in past societies paying attention to the pathocenosis concept introduced by Mirko G. Grmek. Pathocenosis means that the frequency and distribution of every disease depends, on another endogenous and ecological factors, on the frequency and distribution of all other diseases [51]. In consequence, further studies were developed in ancient societies to better know how malaria interact with other diseases causing a considerable decrease in the health status and interact with the constant flow of history. In words of Eric Faure "*Historically, malaria was probably one the diseases with the greatest opportunity to interact with other diseases because of the extent of the malarious areas, of the level of endemicity and of the fact that humans could be infected during all the duration of their lives*" [52].

Malaria can produce positive or negative effects over the diseases in which the illness interacts in direct or indirect forms. As an example, malaria has been applied as a treatment for syphilitic patients. Moreover, Malaria also interacts with tuberculosis (*Mycobacterium tuberculosis*). In this last example, malaria exacerbates the infection and the last one modulates the host response to malaria. It is also important for this research to focus on positive and synergistic interactions of malaria along other diseases that could have deleterious effects in endemic areas. And positive relations with malaria along illnesses that could have a considerable impact on malarial epidemics along diseases such as virus: flaviviruses as dengue or yellow fever; winter respiratory diseases: influenza or smallpox virus. In addition, malaria can interact in positive forms with bacterial diseases such as cholera (*Vibrio cholerae*), plague (*Yersinia pestis*), shigella, and typhus [46] (*Salmonella enterica* serotype *Typhi*) [52]. In words of Eric Fauré, "*Data from the*

pre-antibiotic era suggest that malaria increases the host's susceptibility to invasive bacterial infections" [52].

Finally, in relation to malaria transmission the most complex environmental aspect is found in two principal areas: in *Anopheles* mosquitoes and in sporogonial development inside mosquitoes' stomach. In fact, the link between temperature and oocysts development [53] is essential to know malaria seasonal behavior. As Gustavo Pittaluga said, "*It is not the annual average ambient temperature which determines the degree of endemic (along other conditions), it is more concretely the summer average ambient temperature, the thermal average values of summer, what allows the presence of endemic focus of malaria in temperate climate regions areas*" [54]. In this sense, as mentioned below, through the methodology applied to analyze instrumental meteorological records it varies from minimal temperatures of 17°C for *P. vivax* to 20°C for *P. falciparum* [55, 56]. Our objective is to detect in which years or months malaria epidemics could have a more favorable ambient factor to develop in epidemic forms.

The current problematic for historians' remains in the exactitude to define the variety of Anopheles mosquitoes present in Catalan territories along eighteenth and nineteenth centuries. As a reference, we considered works developed in the first half of the twentieth century such as the Comisión para el Saneamiento de Comarcas Palúdicas (1920–1924) and Comisión Central Antipalúdica (1924–1934) [57]. These works gave us knowledge about the varieties of *Anopheles* that were more common in the Peninsular level until the application of the Proyecto Oficial de Erradicación del Paludismo en España (1959–1962). The works of the zoologist and entomologist Juan Gil Collado inside the campaign led by Gustavo Pittaluga and Sadí de Buen show that from the five varieties that form the complex *Anopheles maculipennis* the variety *labranchiae* was present in Alicante and Murcia. While *Anopheles atroparvus* was the most distributed over Europe [58]. In consequence, we found that our exploratory study focused on examining the relation with environment and *Anopheles* sp. based on the general conditions for "*maculipennis* complex" [59]. Moreover, we analyzed the "superinfection phase" through instrumental meteorological data treatment [60]. The superinfection phase consists of a phase of maximum density of *Anopheles* sp. from April to June. The objective of the study was to focus on those months which present more days with average temperatures less than 25°C [61] and a humidity higher than 40% (fact that was common in all Barcelona area).

One of the possible applications of this historical analysis of malaria over selected Mediterranean areas, regions and cities was to elaborate more precise mathematic models that could help historians to better understand the behavior of the illness in the past. Also, and more important, this analysis could help to develop preventive epidemical models in relation to the current climatic change in which "*Vector-borne diseases are highly sensitive to global warming and associated changes in precipitation*" [62].

3. Sources and methodology

The first question that come to us when we plan to develop studies that involve a high variety and diversity of sources is in relation to which is the most suitable methodology that we must

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apply. The second question concerns what kind of sources we can manage to achieve our objectives. In fact, due to the variety of names that surrounds endemic and epidemic fevers on past societies (terms such as fevers, intermittent fevers, agues, and marshland fevers), we proposed a methodology to study epidemics, from a historical point of view, that would be open to interdisciplinary approaches from history, biology, geography, demography, and climatology. A proposal that reminds us the work of the team lead by Jean-Paul Desaive [63] or the study developed by Vicente Pérez Moreda [64] with a more demographical perspective. Moreover, we can refer to some works published by authors such as Pablo Giménez-Font [65] from the University de Alicante and Mary Jane Dobson [37] from the Oxford Wellcome Unit for the History of Medicine. In addition, the most recent study conducted by an interdisciplinary team from the Aix-Marseille University [55].

Concerning sources, we parted from a general and specified bibliography (papers, monographs, and theses) that allowed us to focus on compiling the highest and possible amount of information.

The amount of sources and their varieties were relatively large: private sources (diaries and memories), public sources (medical reports, contemporary mortality, and morbidity records), municipal sources (books of acts, municipal proclaims, public health reports, and works on hydraulic or sanitarian issues), ecclesiastical sources (burial and baptisms series, and community books of acts), public and private sanitarian institutional sources (medical reports), print sources (newspapers, Royal Decrees, and contemporary medical reports), cartographical sources, instrumental meteorological data (temperature and rainfall records, and wind direction), and flood-drought monthly proxy data indexes generated from Rogation ceremonies (1780–1800) [66].

In consequence, we will emphasize on the principal sources that can be useful to develop similar research over different times and countries, and a methodology suitable to reconstruct other epidemical cases.

3.1. Municipal sources

All the documentation produced by municipal authorities in past societies has arrived to our current times in many diverse forms and ways. This means that we can find more or less complete archives. However, the most common and complete sources that we can find in municipal archives are, e.g., municipal chords, municipal proclamations, and sanitation expedients.

First, the answer to this source selection rests in the serial and continuous data that we can extract from municipal chords as a first source in which all the information relative to the city and quotidian life is reflected. That is, a conjunct of information compiled from notes taken for the secretary at every municipal session, which contains a variety of typologies: proclamations, design of infrastructures, and accounting documents. The utility in relation to epidemiology remains in the keyword research through the contemporary indexes of municipal chords. The objective is to detect every political measure applied to contain epidemics: burial of clothes, latrine cleaning policies, wheat importations, prices regulation of products

affected by commercial blocks or as a consequence of climate instability (carbon, bread, and meat), and police measures applied to guarantee the control of population.

Second, municipal proclamations show the researcher what kind of measures and information were published for the common knowledge, in which data such as urban regulations, advices, prices of essential products, and sanitary policies have a direct impact as measures derived from epidemic situations. After comparing the information extracted from municipal proclamations with the data extracted from municipal chords, the researcher will be able to prove if the municipality had concealed evidences at the time of epidemics.

Finally, sanitarian expedients concentrate all the documents generated or received by the municipal Sanitarian Council. As an example, Barcelona is one of the most complete sanitarian series preserved at the peninsular level with 12 series and 271 independent units providing sanitarian information to the researcher from Barcelona, Catalonia, Spain, and the rest of the world. Although some series are fragmentary, we can obtain data from surveys conducted by physicians, medical records, sanitarian patents, and sanitary chords that can be compared with the information obtained from municipal chords and municipal proclamations.

3.2. Public sources

All the documents from medical institutions such as the Reial Acadèmia de Medicina de Catalunya are another point of interests. The high amount of documentation that we can obtain provides interesting data concerning the symptomatology of the diseases observed at the time of epidemics. In other words, medical topographies conducted by physicians in order to be accepted as partners of the Medical Academy of Barcelona provide interesting data about the common habitudes, landscapes of the past, environmental conditions, and hygienic conditions of the cities analyzed in those works.

On the one hand, medical records were works ordered by municipal authorities in which physicians did an environmental analysis of all the illnesses observed during a concrete chronology. The most interesting aspect we find it in that physicians, following the principles of medical topographies, did a complete study of weather, illnesses, and environmental conditions in every case searching the focus of the illness and the exact time in which epidemics appeared in the cities and villages. A complete work over the urban infrastructures and common people's habits had been corrected through sanitation policy applications.

On the other hand, demographic records collected by physicians in collaboration with ecclesiastic and municipal authorities at the time of epidemics can provide demographic movement of parishes to our research data whose archives are actually inexistent due to the course of history. Another analysis possibility that provides us those demographic tables is to contrast the results with ecclesiastic demographic sources.

Print sources include a high typological diversity and currently the most part is easily accessible through digital repositories. In this sense, the most interesting sources used to complete the information extracted from municipal archives are the medical prescriptions at

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the time of epidemics and published medical records. In their conjunct, these kinds of print sources (considered as primary sources that were coetaneous published to the period of study) can contribute to better understand the social repercussions of an epidemic and the institutional measures applied to spread the information between common people, authorities and physicians. The historical compilations of epidemics as, e.g., Doctor Joaquin de Villalba's work —military physician of Aragon—are more important concerns, where his message goes directly vinculated to the medical pursuit of the historical reconstruction of endemics, epidemics, and epizootic illnesses "*to show taking as an example past situations to better affront future cases*" [67].

3.3. Ecclesiastical sources

The study on the abundant and variety that present ecclesiastical archives makes to focus research interests on the sources that can have a more sense of utility. Parish books are one of the most important sources to develop historical epidemiology due to the high level of detail and trustworthy information that we can extract from parish baptisms, and death and burial records. In fact, since the Council of Trento (1545–1562) all parishes were obligated to establish parish books to register baptisms (births), deaths, and marriages. Those records, currently, are the well-known kinds of sources which worked in the research fields of demography, historical demography and genetics [68].

As discussed above, the amount of information that can be extracted is significant. Although parish registers can present variations from one parish to another, they usually follow a contained structure. The solid structure of parish registers allow us to extract, taking as example baptism records, information relative to baptism date, name and surname of the newborn, parents' names, and parental professional data and born place. While concerning death and burial records, we can extract homogeneous information concerning death date, name and surname of the deceased, parental information, parental professional data, born place and burial place. Since the beginning of the nineteenth century, it has incorporated another interesting data that concern directly to the principles promoted by the hygienist medicine; this is the cause of death.

Obituary tables elaborated by physician Francisco Salvà, from the Royal Academia of Medicina de Barcelona, are good examples of the interest of physicians to improve the cause of death in parish registers to better study the seasonality of illnesses and detect those epidemics that caused elevated mortality levels. In fact, the latter is a good example in which physicians presented the obituary tables. The problematic work in tables was to exactly delimitate the age group through parish registers, "*Must be necessary that from now on in burial records will be expressed the age of deceased, because otherwise it would follow what currently happens with the name of infants must be understood all those that die until the age of 12, 14 or more years, whatever executed with all the other observations from your H.E. of this illustrious city council obituary tables may again be formed same as those currently made in London, Paris or in other places*" [69].

In fact, the problematic work was the precision of the age group qualified in parish sources as infants continue promoting an intense discussion between historical demographers. It is necessary to refer to the studies developed by Vicente Pérez Moreda, especially, mentioning

infants as a collective population comprised of a group of ages from 0 to 7 years old. Moreover, the age group of infants is susceptible, biologically and physically, to succumb to an illness (**Figure 2**) [64].

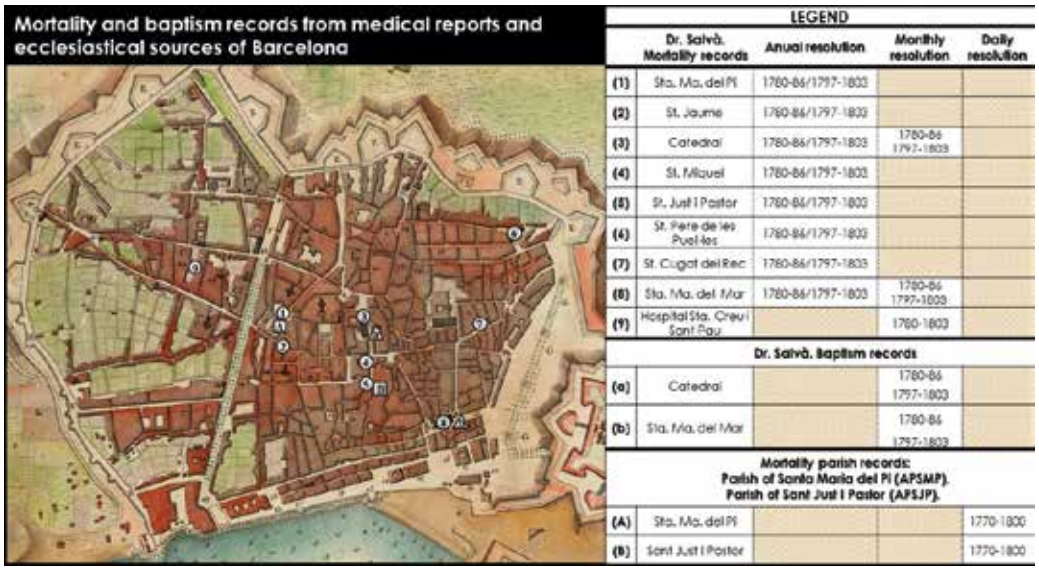

Figure 2. Chronologic extreme dates of demographic sources extracted from parish of Santa Maria del Pi, parish of Sant Just i Pastor, and from the Medical Obituary tables created by physician Dr. Francisco Salvà.

As mentioned above, our interest was to focus on obtaining continuous and detailed data series digitized in excel to realize further analysis that will provide us some interesting information about the seasonality of deaths and the age group that was most affected. As an example, we propose to digitize the information extracted from parish records differentiating sex and age in daily resolution to obtain standardized series in monthly or annual resolution through typification statistical process [70]—among other analytic procedures. Other analytic processes such as seasonal mortality patterns can be of great utility to exactly precise in which season's mortality fall in children or adult population. On the other hand, seasonal movements of mortality can be put in relation to the medical records to have a more complete perspective of the diseases present along with detected mortality peaks.

Finally, parish records in spite of their possible discontinuous or fragmentary preservation can be complemented through obituary tables compiled by physicians. Another possibility to sort the disappeared information from some parish archives lies in coetaneous census conducted by state authorities. As an example, the census conducted by Count of Floridablanca in 1787 shows us in the preface that the scrutiny of Catalonia's population was done "*after three years of an epidemic almost general of tertian fevers and putrid fevers, especially in the two Castillas, Aragon Kingdom, and principality of Catalonia, that has resulted in a considerable diminution of their habitants*" [71].

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3.4. Instrumental meteorological records

Maldà Oscillation [72] becomes the climatologic context that will have a direct impact on the environmental context generating unhealthy area. A process of climatic instability appeared since 1760 until the end of the eighteenth century. A process of climatic oscillation was marked by the simultaneous emergence of droughts, floods, temperature oscillations, and storms. This phase of climatic change, that reminds us of the current climate problematic, as discussed above, has had repercussions over biological and ecological aspects: proliferation of flooded areas and consequences over the hydraulic resources. This phase also had repercussions over the economy: bad harvests, increase of basic product costs because of bad harvests, and effects on the economic resources.

The relation between climate oscillations and the spread of epidemics was one of the principal interests of physicians because of following the principles of hygienist medicine. Even more, as we observed, the pursuit to elaborate a historical relation of epidemics, to observe and measure the weather, to analyze the environment, and the elaboration of accurate compilations of symptomatological descriptions (clinical histories) fructified into a scientific approach of medicine to another sciences were among other interests. This approach encouraged physicians to develop systematic meteorological observations.

Meteorological tables elaborated by physician Dr. Francisco Salvà i Campillo [73] have denoted a great interest to climate reconstruction [74]. In fact, Dr. Mariano Barriendos together with "Team of Climate Change" from the Climatological Area of the Meteorological Service of Catalonia has digitized those tables in a project of three years of duration. The information contained in those meteorological tables, e.g., atmospheric pressure, temperature (measured each day at 7–14 and 22 hours), and precipitation, has a high potential to develop more accurate predictive models due to the current climate change dynamics through initiatives such as MEDARE (MEditerranean DAta REscue) or ACRE (Atmospheric Circulation Reconstruction over the Earth) [75].

What is even more interesting is the inclusion of medical observances that are focused on the epidemical constitution of every month. In those observations, Dr. Francisco Salvà included an accurate description of the symptomatology observed in their patients. Moreover, the physician searched all the references, at the time of epidemics, to find equal epidemic cases in different countries. This completed some information from contemporary sources in relation to reconstruct the specter of endemic and epidemic fevers at European level.

The utility of instrumental meteorological records opens a high range of possible analysis concerning malaria's relation with climate and environment. In fact, the analysis of temperature records will help us to detect which years presented prolonged warm summers or in which months temperature guaranteed a more considerable presence of *Anopheles* mosquitoes. Furthermore, the conjunction of analyzed annual historical indexes of floods and droughts can detect those years in which cities and village's environment was being affected by an irregular succession of floods and droughts that could have reflected a more propitious terrain for the development of a malaria epidemic. These Concern annual-monthly historical indexes those were generated from rogation ceremonies by Barriendos [66].

Although this methodology requires of further detailed analysis methods, we would like to show some analysis to demonstrate the applicability of this sources to help detect the most favorable conditions for malaria development in past societies. Because malaria is a background disease complex to be identified over the sources, we propose to develop analysis from instrumental meteorological records. The objective is to put together all the direct and indirect conjunct of environmental-climatic elements that can be associated with an epidemic of malaria: warm summers, days that present a highest superinfection phase of *Anopheles* mosquitoes (if temperature is less than 25°C between April and June and humidity is higher than 40%, those years present a large number of *Anopheles* sp.), favorable days for sporogony of *P. vivax* and *P. falciparum* based on the minimal daily temperatures of June-October for all the available and monthly minimum temperatures of June-October for all the period observed (1780–1800).

4. The epidemic context in Catalonia (1783–1786)

The report from Real Tribunal del Protomedicato published in 1785 detailed the constant presence of malaria at Peninsular level "*Tertian fevers have been ever in Spain the dominant illness, and for this reason our authors are the most respectable between the strangers, giving us a clear idea to differentiate over all the forms over tertian fevers those that currently are affecting us*" [76]. This epidemic of malaria, since 1783, is identified in epidemic forms outside its hyperendemic areas (Valencia and Catalonia rice fields and marshlands) along other diseases as typhoid fevers. Tertian fevers and putrid fevers were in fact well known through the Mediterranean littoral areas. Even more, contemporary physicians such as Andrés Piquer noted the strong relation between tertian fevers and rice cultivation or irrigation patterns insisting to the authorities to implement the prohibition of rice cultivation to one league of distance from urban areas. However, the high interests of land proprietors to avoid prohibition or reconcile the legislation to make possible the continuation of rice cultivation was a constant problem (**Figure 3**) [25].

However, tertian fevers epidemic of 1783–1786 could not be compared to precedent equal episodes. In fact, authors such as Vicente Pérez Moreda elevate the impact of the epidemic to a one million affected people and a hundred thousand deaths just in 1786. Moreover, epidemic fevers will spread accompanied by a favorable climatic oscillation and scarcity periods. In words of Pablo Giménez-Font, "*it's possible that a conjunct of favorable conditions, mainly climatic conditions, increased tertian fevers range of impact from hyperendemic areas, such as Valencian ricefields or marshlands close to Cartagena*" [65]. In fact, the relation between the climatic oscillation and the genesis of epidemic fevers was well perceived by contemporary physicians linked to the Real Academia de Medicina de Barcelona as can be seen through the testimony of doctor Juan Tovares "*Too much rain, fogs and snows what they had to give us but rafts, puddles and lagoons, floods, water spills and stagnant waters in embanked landscapes? All this humidity altered vegetation so that fruits, although they were abundant, their quality decreased becoming rot much easier than before* (…) *propagation of bugs, mosquitoes, and other insects was amazing* (…) *to this abundance of water in the autumns, winters and springs succeeded the heath of summers, that dissipating the stagnant waters from puddles, lagoons and rafts formed marshes and quagmires filled the atmosphere of vapor and putrid*

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miasmas elevated from the high amount of rotten vegetables and putrefied vermin, and those were the remote causes of the referred epidemic" [77].

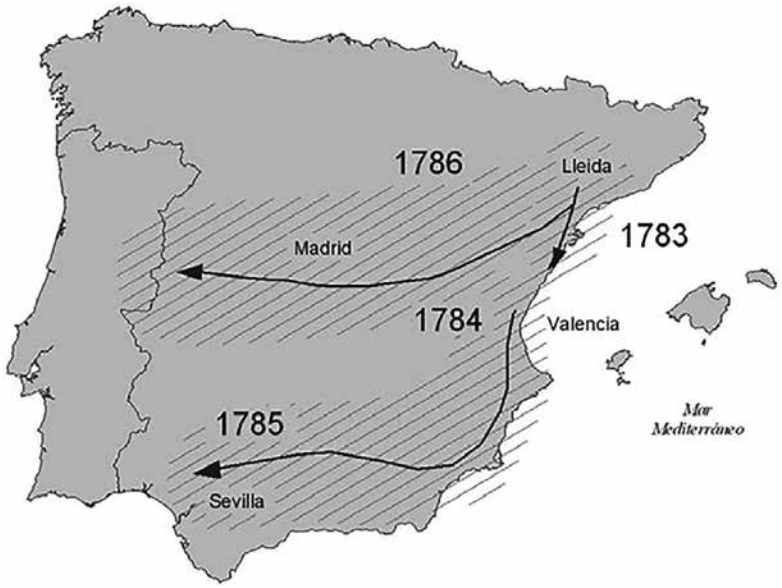

Figure 3. [Extension of tertian fevers epidemic at peninsular level (1783–1786)]. Giménez-Font [65].

Tertian fevers from 1783 onward spread over West, South, and South East of Spain at a peninsular level after being initially declared in Lleida (Catalonia, NE Spain). More importantly, since 1783 until 1785 the principal affected areas were Catalonia, Valencia (SE Spain) and Murcia (SE Spain). While in 1786 the regions that suffered the most damaging effects were Andalusia (South Spain), Castilla la Mancha (South West Spain), and North Sub-Plateau (North center Spain). Once more, physician Juan Tovares pointed that "*by the years 84 and 85 it was especially cruel* [fevers epidemic] *in 86, devastating the major part of our peninsula; but moreover provinces of la Mancha and Alcarria, leaving some villages reduced to a few habitants*" [77]. Fevers with symptomatology, as can be appreciated through the testimony of doctor Christobal Cubillas from Cafez, were commonly detected as a "*fever more or less high with a day of duration. In some cases fevers were extended to two days, just a very few arrived to the third day* (…) *the fever regularly finished by sweat, and if afterwards sweating continued, this was the greatest success and the lesser evil*" (**Figure 4**) [67].

On 18 May 1783, the message in the letter sent to the physician Josep Masdevall by Floridablanca's Count was explicit. The work entrusted to Masdevall elaborated a memory that contained all the news taken by the physician in relation to the epidemic that spread in Catalan territories since 1783, an epidemic that "*since early past year was discovered in the city of Lérida, spreading through all Urgel plain, Conca de Barberá, fields of Tarragona, Segarra, Manresa, Llusanés, Solsona, until Seu de Urgel and their surroundings, spreading strongly through Igualada, Piera, Vilafranca del Penedès, Martorell and another nearby towns*" [78].

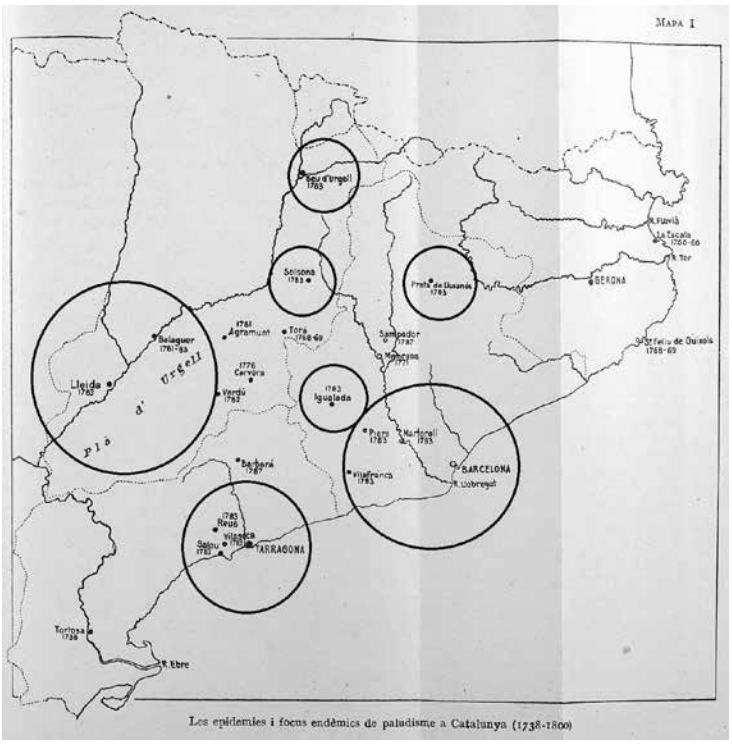

Figure 4. Geographic map elaborated from ancient sources in which is represented the most important epidemical focus of tertian fevers detected in the last years of 18th centuries. Institut de Ciències. Treballs del Servei Tècnic del Paludisme: 1915–1916. Barcelona: Publicacions del Institut de Ciències. 1918, pp. 38–41, 156 p.

In spite of the efforts put by Royal Sanitarian Joint of Barcelona and Royal Sanitarian Joint of Madrid, the efficacy of the lack of practices and remedies stipulated by them did not control the epidemic. Those remedies did not reduce the virulence of the epidemic affected by the illness "*sturdiest people from twenty to fifty years old*" [78]. These epidemic fevers had their origin, following Dr. Masdevall opinion, in the French retry from Portugal in 1764, in the context of the end of the Seven Years' War "*we must confess, that since the retry of French troops we suffer of more malignant fevers and agues than before*" [78] probably favored by the initial climatic oscillations of 1760. Moreover, the retry of French troops supported the opinion of Masdevall that the increased virulence of a preexistent problematic spread silently from one village to another, "*The communication, commerce, friendship and relationship of people's from transit of French troops with the remaining in which they didn't passed, communicated also to them the referred injuries*" [78].

Epidemic fevers that "*started with a sensitive cold, followed by an intense heat, which disappeared through an excessive sweat… Headache was really intense… others presented a bulky abdomen… hand shaking, or convulsions*" [78]. In fact, the epidemic cases detected threatened through the application of the "Antimonial mixture" that caused vomit and increased transpiration, followed by the use of "Opiata" (a chemical remedy in which quine is the principal ingredient).

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The relative success of the remedy applied by Masdevall and their efforts were compensated on 30 October 1783 when King Charles III communicated through Count of Floridablanca his promotion as royal physician and Inspector of Epidemics of Spanish kingdom with a salary of 20,000 reals [79].

5. The medical reports of 1783–1786

Through this point, we focus our attention on the interest of physicians to find the origin of epidemic over the most common infectious and proactive areas of Barcelona. Moreover, in both medical reports we find a transition in relation to the search of the environmental cause effect of epidemics and over human productive activities, urban resources… The objective of physicians is to promote a systematic application of hygienic policies to prevent future epidemic situations.

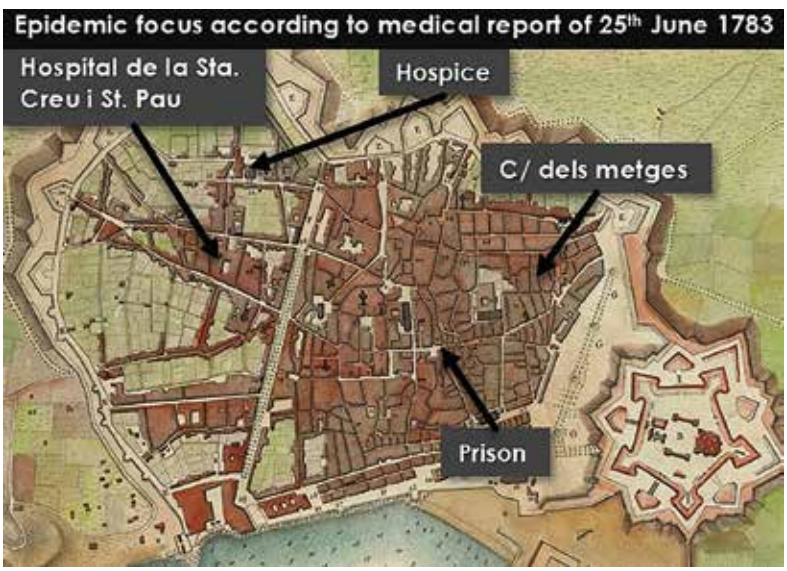

Figure 5. Self-elaborated map from the information provided in the medical report of 25 June 1783. MOULINIER. Plano de la ciudad y Puerto de Barcelona. 1806. ICGC, RM. 19425.

On 19 May 1783, the City Council of Barcelona alerted the presence of tertian fevers in his more immediate areas to the Local Joint of Sanity "*With date of the current 19 may* [1783], *the City Council of this city assembled in Joint of Sanity show: That some of the surroundings of this Capital reign some diseases that we suspect they are similar or maybe the same illnesses that reign in the part of Lérida*" [80]. In consequence, physicians Buenaventura Milans, Gaspar Balaguer, Pere Güell (Protomedicate tenant), Rafael Steva (Doctor in Public Health), Pablo Balmes, Luis Prats and Benito Pujol elaborated a medical report following the orders of the Supreme Joint of Sanity and the Local Joint of Sanity of Barcelona. A medical report focused on the continuous fevers

that spread in Barcelona. More importantly, these fevers were specifically detected on "Metges" street, the prison, the hospice, and the Hospital of Santa Creu. In spite of the research effectuated in the most proactive places and areas to be the focus of the epidemic, physicians rejected both houses because before the epidemic was present in the surrounding villages. This illness affected the income of a large number of people of Barcelona's City Hospital (**Figure 5**).

Continuous fevers that "*are currently abundant between poor people of Barcelona, many soldiers, and they become to be present between a few well of people*" [81] with an initial symptomatology quite similar to flu or cold. But the major part of fevers became putrid fevers and, moreover, some of them became malign fevers leaving the major part of the patients prostrated in their beds. Succeeding to the first clinical symptoms of the disease sweat and delirium, "*they become to be delirious, when we least expect it the major part of patients die*" [82]. The relation between tertian fevers and climate-environmental conditions focused on the medical report making reference in the form of benign illnesses that were common in spring and autumn that used to disappear with the summer months. Nonetheless, in 1783, physicians confirmed that these were fevers outside of their seasonal behavior with a considerable preoccupation of doctors who speculated about the possibility of a more increased virulence of fevers during the summer, "*in Lerida fevers have been common as well as in cold or hot seasons. We suspect that with the hot of the terrain will grow up illnesses, which had started to be popular at the arrival of hot weather*" [83].

The pursuit to detect the epidemic origin was the principal objective for the physicians as was mentioned in the records of the first patient who died in the Hospital of Santa Creu. Felip Pujan, who was a soldier of the Royal Walloon Guards, deceased on 21 July 1783 because of typhoid fevers that suspects the possibly of the coexistence of malaria along with typhoid fevers that is denoted as the virulence of the epidemic in the observed period.

One of the interests of physicians was the will of redirecting the hygienic habitudes of inhabitants especially of poor people "*The poor that are abundant in the streets because of the high misery of the Principality. The poverty that can't be repaired because of the consecutive bad harvests. The poor people have been in the major part the victims of the disease* (…) *this poor day laborers and artisans, in which is the epidemic is abundant, live in wretch rooms. An only room contains a high quantity of dirty beds, a kitchen, a dining room, and everything. Latrines are a conduct in the same room that is never totally clean of excrement and that throw out an unpleasant smell*" [84].

About tertian fever epidemic, physicians proposed the application of a curative methodology essentially based on the systematic application of bleedings, diuretics, emetics, and purges to expel from patients' body all infective material. The most probable effect of this kind of medical treatment in patients affected by malaria could have a silent impact on the public health of the inhabitants of Barcelona. Although had the knowledge of febrifuge attribute of quine, this remedy was only applied in those cases in which fever was really outstanding or if the previously exposed remedies were not completely effective. Otherwise, the use of bad quality quine could not have the expected effect on the related fever epidemics. Moreover, the discussion between traditionalist physicians and chemical remedies will be a constant problem.

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Finally in this first medical report, physicians provided a conjunct of hygienic policies that should be applied to preserve the health status in the city and to face on future epidemic cases. These initiatives were specifically made to increase the healthiness level of the city, e.g., stagnant waters of Montjuic and Besós River should be systematically drained on critic epidemic episodes. On the other hand, doctors will focus their interests in helping the authorities to guarantee a better quality of bread increasing alimentation quality of the city inhabitants.

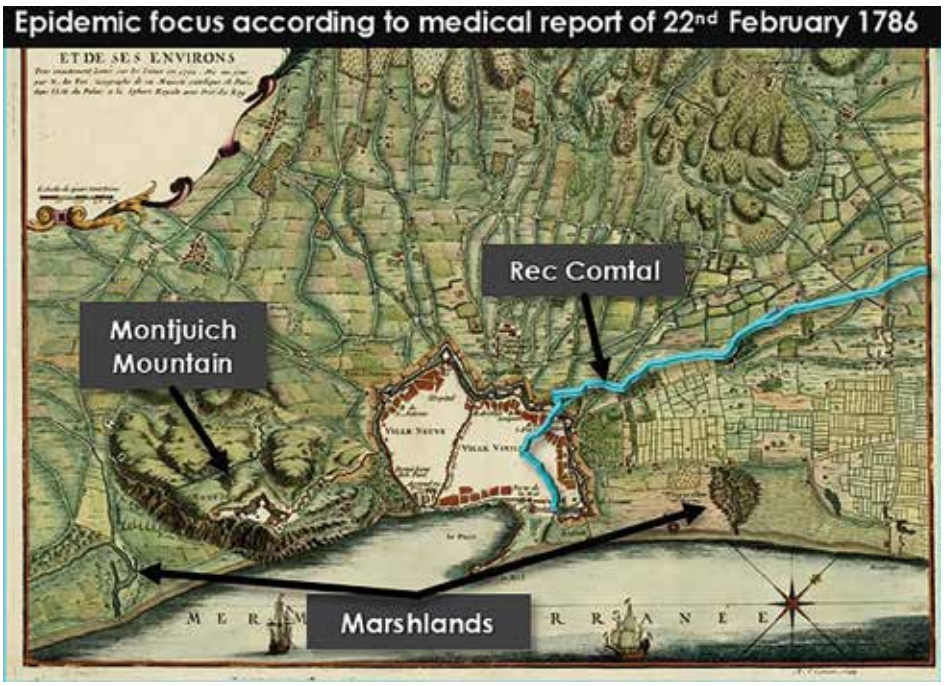

Figure 6. Self-elaborated map from the information provided in the medical report of 22 February 1786. FER, N. Plan de Barcelone et de ses environs, très exactement levés sur les lieux en 1711. Paris: dans l'ile du Palais, à la Sphère Royale. 1711. BNF, GED-1695.

The second medical report presented on 22 February 1786 was another point of interest in which we focused our attention on tertian fever epidemics in Barcelona. This time physicians Rafael Steva, Pablo Balmes, and Lluis Prats answered the order given by Count Campomanes who instigated municipal authorities to spread information that could explain the progression of tertian fevers epidemics in 1785. In the first assessment done by the physicians they admitted that tertian fevers were unusual but more common than in previous years "*but being general in this year tertian fevers over all Kingdom, the fevers have been very rare in the city, but more frequent than in other years*" [85]. But the most preoccupant point was tertian fevers that evolved progressively from benign fevers to putrid fevers "*Have been also particular that inside Barcelona have become malign fevers some that were originally simple, and they keep as such, and benign in some cases. The major parte have been putrid, some of them mixt*" [86].

In this medical report physicians explained the origin of the epidemical cases with an emphasized focus on the irregular climatic behavior of seasons. More importantly, physicians denoted the climatic alteration over the usual behavior of months from June to August. As expressed in physicians' words, "*This year has been particular in the irregularity of the seasons, and being the month of June very heat July and August have been very temperate, rather said fresh, being particular the heat diminution that is noted over our meteorological tables, what maybe is the cause of the major frequency of tertian fevers this year*" [87]. But this time, the origin of the epidemic directly searched upon the environment. That is to say, over the immediate unhealthy areas that surrounded Barcelona in the late eighteenth century.

In consequence, physicians declared Ciudadela, Montjuich Mountain, and Rec Comtal (principal water supply of the most crowded and industrial quarter of Barcelona) and the cotton factories in "Prados de Indianas" and marshlands which is consisted of Barcelona's periphery as unhealthy areas (**Figure 6**).

From the areas exposed above, physicians emphasized to focus authorities' attention on the dangers emanated from stagnant waters. As an example, physicians mentioned the case of the suburban area known as "raval." Because this area fall into the urbanized area of the city it was "*actually very healthy, after having given course to waters that used to be stagnant in the pit that goes from the Puerta de San Antonio to Puerta de Santa Madrona*" [88]. Another interesting case was related to the Ciudadela fortress due to the stagnant waters that were present over all their pits "*and will not be hard to understand why Ciudadela is not healthy, being enough maybe that their pits are double sized, and they are extremely flat that the water remains stagnant* (…) *furthermore we know that inside the fortress there are too much garbage heap which poison the air*" [89].

Moreover, as discussed above, the attention of physicians was focused on the unhealthiest spaces that surrounded the urban area. Insalubrious spaces conditioned the normal development of the city. In fact, Barcelona was a city marked by an infectious suburb in which tertian fevers wreaked upon the civil population generating a constant flow of ill people to the Hospital of Santa Creu. Although there were more the healed than the dead because of the disease, this could have an impact on the presence of indoor malaria between the city walls.

What is a good example are the words that are mentioned in the medical report. Words that denote a high preoccupation of physicians were in fact an illness presented on inhabitants' quotidian life "*Could we look with indifference what means to be surrounded by a source of epidemics in both marines, to be unable to leave to our country estates, to not be able to continue our factories without seeing a constant loose over their offices, their meadows, a high amount of workers. Family men of families that remain orphans. Factories owners that have to rest in the city being not able to go to the offices of their factories when is sometimes interesting to request their presence?"* [90]. In fact, the epidemic focus in this last medical report will be clearly specified on both marines "*It's not difficult to find the origins of insalubrity because both marshlands are insane* (…) *in those marshlands stagnant waters become corrupt when arrives the dry weather* (…) *cane cultivation rafts are really numerous as a consequence of not being followed the hygienic measures that we had exposed to H.E.*" [91]. Those unhealthy areas were not only a consequence of their environmental particularities, but also reported the anthropic impact, the productive activities such as cane cultivation rafts that confer to these areas the meaning of being insalubrious and dangerous for human health.

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Because of the problems discussed above, the preventive hygienic measures stipulated on the study directly focused on the periphery of the city, especially concerning hydric resource management. Thus, doctors emphasized the necessity of building more ditches to reroute the stagnant waters present in the coastal region to make them flow in the sea. More importantly, physicians proposed to completely drain both marshlands that surrounded Barcelona "*But above all should be drained both marshlands Remolà and Port, with what is more probable would be very healthy Montjuich"* [92].

Finally, physicians agreed on to follow the medical prescriptions given by the Real Tribunal del Protomedicato. From now on, bleedings were totally avoided in favor of a treatment related to fevers based on the administration of consistent doses of quine and laxatives.

6. The obituary tables from Medical Academy of Barcelona (1783–1786)

Because this is an ongoing research, we would like to show some results of the analyzed mortality series of Barcelona. Due to the fragmentary mortality series obtained through obituary registers from the two of the three currently preserved parish archives of Barcelona (Parish of Santa Maria del Pi and Parish of Sant Just i Sant Pastor), we use as an introduction the mortality records extracted from necrological tables created by physicians of the Real Academia Médico-Práctica de Barcelona. Through this analysis we would like to show some detailed annual results especially focused on detecting parishes that concentrate on the most outstanding children mortality peaks (**Figure 7**).

Through the displayed graph from 1783–1786, we can clearly appreciate the years that presented an anomaly concerning mortality peaks, specifically 1781, 1783, 1785, and 1786. In 1781, in general, burials increased with respect to the previous year. In fact, child mortality exceeded adult mortality in eight Barcelona parishes. In this sense, Barcelona's Cathedral reflects 893 children in contrast to 159 adult people deceased. Moreover, it will be the traditional commercial-artisanal area that will remain the second most elevated value of childish mortality. More importantly, it is the parish of Santa Maria del Mar with 500 children versus the 208 adult bodies registered.

On the other hand, in 1782, mortality over all eight parishes of Barcelona was still being elevated (2724 deceased). In fact, the mortality increment in all parishes of Barcelona is clearly noticed, including the Cathedral, with respect to the previous year. In fact, in the Cathedral child mortality peaks were clearly high with 853 child faced to 168 adult. The conjunction of catastrophic flood events along elevated minimum temperatures and a large number of favorable days for plasmodium development maybe the possible explanation to this continuous over mortality. The constant increase in adult mortality culminates in 1783 with a value of 1.26 standard deviation with respect to the rest of corpses deceased for this series of mortality recovered data. However, although the general lines of mortality are less than in 1781 and 1782, this is because of child population which led the deceleration of the general mortality in 1783. In fact, in 1783 adult corpses surpassed child mortality in all Barcelona's parishes. The exceptions were the parish of Sant Pere (103 adults and 137 children), the Cathedral (192 adults

and 700 children), and the parish of Santa María del Mar (299 adults and 308 children). The adult mortality and children mortality that could be in a strong relation to the impact of typhoid fevers together with the presence of endemic and epidemic malaria could have been entered to the city by the affluence of ill people from the suburban areas.

A new minimal mortality peak reappeared in 1784 (2340 total deceased) with almost one standard deviation less than the rest of the mortality values—typical deviation of minus 0.95. The values that proceed after a diminution of childish mortality—typical deviation of minus 0.97—in conjunction with adult mortality—a typical deviation of minus 0.01.

The year 1785 concentrates the maximum conjunct of the absolute mortality values (3276 deaths) with adult and children manifesting the same upward trend in mortality. In this sense, the intensity in which children mortality exceeds adult corpses due to the negative maximum typical deviation of 1784 in 1785—typical deviation of 1.75—is especially noticeable.

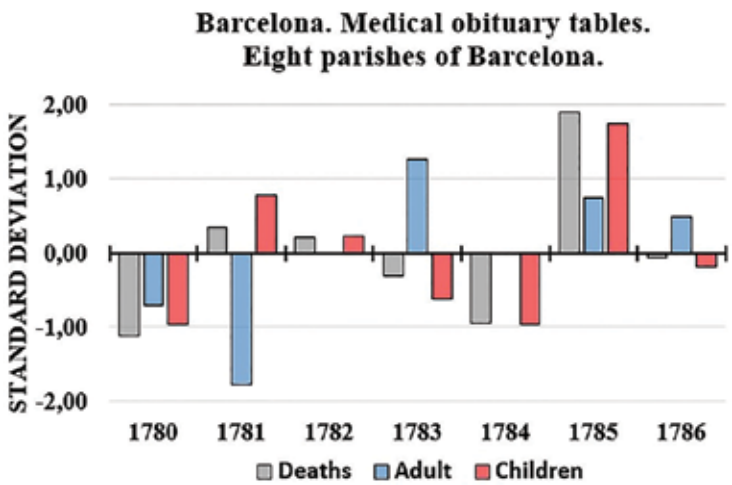

Figure 7. Mortality records of Barcelona in which are reflected the adult and childish mortality from the eight parishes of the city since 1780 until 1786. ARAMC, "Papeles del Dr. D. Francisco Salvà," legajo XII, num 1, "Notas para las tablas necrológicas…," doc. 9. "Muertos en Barcelona, por parroquias, años 1780 a 1786, 1797 a 1800 y primeros meses de 1801, 1802 y 1803."

Childish mortality in 1785 is uniform in all the parishes of the city. However, the most noticeable parishes where mortality wreaked havoc upon the civil population were the Cathedral (160 corpses, 959 children), and the parishes of Santa María del Mar (291 corpses, 528 children), parish of Santa María del Pi (267 adults, 319 children), and Sant Pere de les Puel les (100 corpses, 222 children). The conjunction of minimal monthly temperatures higher than 22.5°C, a high number of favorable days for sporogony of both analyzed plasmodium, and the critical rain episodes that succeeded since 1782 and 1785 could explain the anomaly in childish population as a consequence of a more favorable environment to the development of malaria. This disease could have been presented in a more prolonged seasonal pattern until the autumn months. In 1785, in the month of July physician Dr. Francisco Salvà noticed a transition based

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on a more aggressive presence of putrid fevers: "*In the month of July, the constitution was less inflammatory but more putrid than in the previous month* (…) *tertian fevers were very common this month. Were also detected colic pains and convulsions that were attached by the use of narcotics. In August the epidemical constitution didn't changed, with the exception of some small-poxes, and tertian fevers, there were not more noticeable diseases; but were common diarrheas, dysenteries*" [93].

Finally, a significant high values of child mortality is observed in 1786 in all parishes of Barcelona. In fact, the only exception was Sant Jaume parish (43 adult, 26 children). Children who had the more reduced opportunities to survive due to the combination of malaria along with gastrointestinal diseases "*Those weaning children had the less favorable time. Some of them were affected by a universal weakness after which they died*" [93].

7. Discussion and conclusions

Malaria is a protean disease very sensitive to changes in precipitation, temperature, and wind direction. As shown above, the strong relation between the disease and environment is linked by the principal vector, *Anopheles* female's mosquitoes that give to the disease a high range of territorial affection. Since the progressive disappearance of the plague from European countries, endemic malaria plays a role as a background illness due to the synergic, positive, and negative interactions among other sicknesses such as smallpox, flu, cholera, yellow fever —among others—generating a constant oscillation in mortality peaks. However, historians usually have not paid the merited attention to an illness that was anchored in the quotidian lives, troop movements, the economy, and the landscapes of past Europe since the sixteenth century until the systematic eradication of malaria in the second half of the twentieth century. This illness caused deleterious effects on the health status of peoples of the past, being the children, the newborns were the principal affected collective. This sickness was indirectly fed by those parents who fought for the systematic construction of irrigation trenches to ensure a good alimentation and guarantee a minimum salary for their families.

Malaria is a complex disease with multiple facets and symptoms. In fact, the complexity of the illness resides in the high range of aspects in which it can have an impact on ancient societies. In consequence, the problem to develop historical studies focused on the detection of endemic and epidemic malaria that can only be solved with an interdisciplinary research that will involve a high variety of research fields. One of the most problematic aspects that we usually find in our research was to correctly identify each disease. Due to the above reason, the methodology that we propose is based on to conjunct and compare the most complete range of sources that we can manage. As shown above, from the bibliography we can part a solid basis for the problem to later investigate our corpus of sources. A first step would be the in-depth study of a selection of municipal sources, essentially, municipal chords and municipal proclaims. This step will provide us a rich documentation of quotidian life, hygienic policies, and essential product supplies, all the negative consequences derived from climatic instability and the economic status of the city and the repercussion over the economy as a consequence of the epidemic context. The information extracted from municipal chords can be reinforced

with the help of sanitation sources, such as sanitation expedients. The fact to cross and conjunct all the information extracted from municipal sources will provide us an exact chronology of the effects and the perception of epidemics in the past societies.

Public sources such as the documentation extracted from Reial Acadèmia de Medicina de Catalunya show the potential to realize deepest research over medical archives. In fact, from medical records and instrumental meteorological data we detect a high range of interesting sources such as medical topographies, which in their conjunct and confronted to the municipal sources or the information contained in print sources can contribute to an exact description and analysis of the symptomatology detected in epidemics and hygienic measures proposed by the physicians to municipal authorities and state institutions related to epidemics. Instrumental meteorological records open a high range of possible analysis concerning vector or virus diseases. In fact, one of the current problems is to develop precise mathematical models that cannot help historians to better detect the territorial affection of the diseases but also their seasonal behavior. One of our personal interests is to develop the analysis of instrumental meteorological data with the support of mathematics, physics, entomologists, and biologist to further develop the most detailed analysis in our next studies.

Ecclesiastical sources, more importantly baptism records and burial records, deliver to the researcher the possibility to contrast the information with the corpus sources. More important is the possibility to detect mortality peaks that go unusually high and far from their seasonal patterns of mortality. These mortality peaks can also contradict the symptomatology detected by contemporary physicians for a concrete time lapse and can also be put in relation to the resultant analysis of the environmental requisites for vector-borne diseases such as malaria. As an example, unless we cannot say that all childish mortality peaks are clearly caused by malaria, we can undoubtedly detect the presence of the illness associated with gastrointestinal diseases, typhoid fevers or smallpox, among other diseases.

In the late eighteenth century, we found an interesting conjunction of socioeconomic conditions, environmental conditioners, and urban structures that were, in their conjunct, submitted to the historical context derived from the Spanish Succession War. Since the new political order resultant applied the "Real Decreto de Nueva Planta," the pre-existent Catalan sanitation and political institutions were reshuffled to respond to the interest of the Bourbon monarchy. At the end of the eighteenth century, cities such as Barcelona adapted to political changes but were also submitted to the consequences derived from the new political order. In this sense, Barcelona was under a massive income of population that arrived to the city due to the stagnant agricultural patterns of Catalonia in the middle of the eighteenth century. This city offered the possibility to export agricultural surplus due to the important commercial flow and also the opportunity to have a job in the industrial-artisanal areas of the city. The hygienic repercussion of the city with almost 114,100 population fell over the hydric structures that could not be properly adapted.

On the other hand, municipal authorities could not guarantee an appropriate hygienic management of hydric resources due to the drastic reduction of the City budget and the lack of municipal competences. Water that flows through the city carries the miasmas, all the residues of the industrial areas, and the mix of fecal matters with drinking waters. Moreover,

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the water that came from the Besós River through the channel known as Rec Comtal was the principal cause of contamination due to the traditional presence of legal and illegal cane cultivation rafts.

The relation between stagnant waters and the presence of tertian fevers was well known by physicians and municipal authorities. Barcelona is a good sample that can clearly appreciate through the medical reports presented about the tertian fevers epidemic context that took place between 1783 and 1786. In both medical records, it is clearly detected that the physicians searched the relation with environment and diseases, first inside the city but thereafter doctors refocused their research on the suburban areas of the city and more importantly in both marshlands.

In fact, in the late eighteenth century sanitation institutions were clearly a reminiscence of the pre-existent health structures before the sanitarian institutional reshuffle that took place in 1720. Physicians, in spite of the consequences of the new Bourbonic order, had a considerable impact on the decisions in hygienic policies applied by the municipal authorities. We found a good example which shows that many proclamations detected between 1783 and 1786 focused on the management of marshlands and lagoons that surrounded Barcelona.

However, the dramatic loss of prestige and control over medical studies would be the principal incentive in the constant pursuit of a medical academy in Barcelona. The medical academy since its foundation on 2 July 1770 will rigorously apply and follow the principles of hygienic medicine instigating all the physicians of the medical academy to develop historical medical studies to better understand the causes of illnesses, the relation with environment, the weather, and the sickness.

Acknowledgements

This work is produced within the framework of the Unit of Excellence LabexMed, Social Sciences and Humanities, at the heart of multidisciplinary research for the Mediterranean, under grant number 10-LABX-0090. This work is supported by a state grant administrated by the Agence Nationale de la Recherche for the project Investissements d'Avenir A*MIDEX under grant no. ANR-11-IDEX-0001-02.

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Chapter 2

Transyears Competing with the Seasons in Tropical Malaria Incidence

Lyazzat Gumarova, Germaine Cornelissen,
Borislav D Dimitrov and Franz Halberg

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64332

Abstract

Communicable and non-communicable diseases show coperiodisms (shared cycles) with the sun's and earth's magnetism. About 11-year cycles and components with periods a few weeks or a few months longer than one year (near- and far-transyears, respectively) are the cases in point. Published data on the incidence of malaria in Burundi, Papua New Guinea, and Thailand are analysed by the linear-nonlinear cosinor to assess the relative prominence of transyears versus the calendar year. An about 2.3-year component characterizes malaria incidence in Burundi and Papua New Guinea (Thailand data were only sampled yearly). Long-term trends cannot be distinguished from the presence of an about 11-year cycle found in a 100-year long record from Chizhevsky on mortality from cholera in Russia, albeit its second harmonic is statistically significant in Burundi's data. Whereas far- and near-transyears characterize malaria incidence in Burundi more prominently than the calendar year, only a candidate near-transyear of small amplitude is barely detected in Papua New Guinea, where the calendar year is most prominently expressed. Both regions are located near the equator. Selectively-assorted geographic differences such as these, observed herein for a communicable disease, have been previously observed for non-communicable conditions, such as sudden cardiac death.

Keywords: Chronoepidemiology, Malaria, time series analysis, public health, infectious diseases

1. Introduction

Signatures of cycles in the sun's and the earth's magnetism, found in the aetiology of both communicable and non-communicable diseases, are selectively assorted geographically [1]. Far-transyears and near-transyears (components with periods a few months or a few weeks longer than the calendar year, respectively) are both known to characterize interplanetary and terrestrial magnetism and their biospheric signatures [1–3]. A geographic study of the incidence of sudden cardiac death reveals the prominence of the transyear over the calendar year in Minnesota and Tokyo, whereas the opposite holds in Hong Kong, North Carolina (USA), and the Republic of Georgia [2, 4].

About 11-year (Horrebow-Schwabe) cycles of relative sunspot numbers [5–7] have also been reported to characterize communicable diseases such as malaria, as suggested earlier by Dimitrov et al. [8]. In their analysis of cerebral malaria in Papua New Guinea (latitude between 0° and 12°S, longitude 140°–160°E), these authors also reported the presence of a strong calendar-yearly component, in the absence of a marked transyear. By contrast, a transyear over calendar year prominence at -3° from the equator characterized a time series of natality in Mindanao, Philippines (8°N, 125°E) [2,3]. In a study of malaria in Thailand, a period of about 4 years was prominent, while geographic differences prevailed; "seasonal" cycles were only synchronous in small clusters [9].

Gomez-Elipe et al. [10] forecast malaria incidence based on monthly case reports and environmental factors in Karuzi, a province of the Burundi highlands (at 3°6′5″ S, 30°9′53″ E), recorded from 1997 to 2003. The analysis is complicated by the occurrence of a large outbreak, resulting in outlying values. The authors developed a satisfactory model to predict malaria incidence (notifications of malaria cases from local health facilities) in an area of unstable transmission by studying the association between environmental variables (rain, temperature, and a vegetation index, NDVI) and disease dynamics. Their autoregressive integrated moving average model predicted malaria incidence during a given month based on the incidence during the previous month together with NDVI, mean maximum temperature, and rainfall during the previous month with a 93% forecasting accuracy (R^2adj = 82%, $P < 0.0001$). While their model was useful for forecasting the malaria incidence rate in the study area, we ask whether cycles in malaria incidence found in Karuzi are similar to those found in other geographic sites located near the equator, where populations are exposed to factors that strongly influence the origin and magnitude of malaria epidemics, such as a weakened immunity of the population associated with famine and massive displacements, failures of control measures and epidemiologic disease surveillance, and unstable environmental factors such as rainfall, temperature, and vegetation [11].

Herein, we revisit published data on the incidence of malaria in different geographic locations near the equator. For this purpose, we turn to data in Papua New Guinea [8], Thailand, and Karuzi, Burundi [10], to explore any differences in terms of the relative prominence of transyears versus the calendar year. Our meta-analysis raises the question whether space weather may also contribute to communicable diseases like malaria in certain geographic

http://dx.doi.org/10.5772/64332

regions at certain but not at other longitudes and/or as a function of prevailing climatologic factors.

2. Materials and methods

Data from Papua New Guinea consist of the total monthly admissions for cerebral malaria and of those from selected facilities, recorded between 1987 and 1996 [8]. Data from Thailand are those reported by WHO (www.sears.who.int/EN/), available yearly between 1971 and 2010 for the percentage of microscopically diagnosed malaria positive slides found in blood smears examined, the number of cases of *Plasmodium falciparum* infections (including mixed infections), the number of *P. falciparum* infections per 100 slides examined, the percentage of *P. falciparum* infections per 100 malaria positives, and the number and percentage of malaria-related deaths, among others. Data from Karuzi, Burundi, are those published by Gomez-Elipe et al. [10], available from 1997 to 2003.

Data were analysed by the extended cosinor [12–15]. Least squares spectra examined the entire time structure (globally) to identify candidate cycles as spectral peaks. Nonlinear least squares based on Marquardt's algorithm [16] provided estimates of the periods involved with a measure of uncertainty as "conservative" 95% confidence intervals (CIs).

3. Results

After square root transformation, the monthly incidence of malaria in Burundi was characterized by a near- and far-transyear, and by components with periods of about 5.2 and 2.3 years. Results from the nonlinear analyses are summarized in **Table 1**. The corresponding model fitted to the data is illustrated in **Figure 1**. As illustrated in **Figure 2** (left and middle), the near- and far-transyears with periods of about 1.15 and 1.5 years, respectively, are more prominently expressed than the 1.0-year synchronized (calendar) component, as gauged by amplitude ratios.

The about 5.2-year component found in Burundi (**Figure 1**) may correspond to the second harmonic of the decadal cycle reported by Dimitrov et al. [8] for cases of cerebral malaria in Papua New Guinea. A component with a period slightly longer than 2 years was also reported by Dimitrov et al. [8] for the data in Papua New Guinea, together with a prominent yearly rhythm, as seen from least squares spectra of the original data and of the detrended data, obtained by removing either a linear or a quadratic trend (**Figure 3(A)–(C)**). The large-amplitude low-frequency component reflects a trend, a low-frequency cycle, or both, which may be difficult to separate in view of the brevity of the series. There is also a smaller spectral peak (below the noise level) that may correspond to a near-transyear, perhaps the second harmonic of the slightly longer-than-2-year component.

All components fitted concomitantly					
Period	**[95% CI]**		**Amplitude**	**[95% CI]**	
Original data					
4.78	3.89	5.68	7.04	3.68	10.40
2.26	1.95	2.58	4.80	1.38	8.21
1.50	1.34	1.66	4.82	1.44	8.19
1.13	1.02	1.24	3.90	0.63	7.17
After square-root transformation					
5.18	4.21	6.15	1.05	0.65	1.45
2.34	1.93	2.74	0.55	0.14	0.96
1.54	1.35	1.73	0.59	0.17	1.00
1.15	1.04	1.27	0.50	0.11	0.90

Table 1. Nonlinear results of monthly incidence of malaria in Burundi.

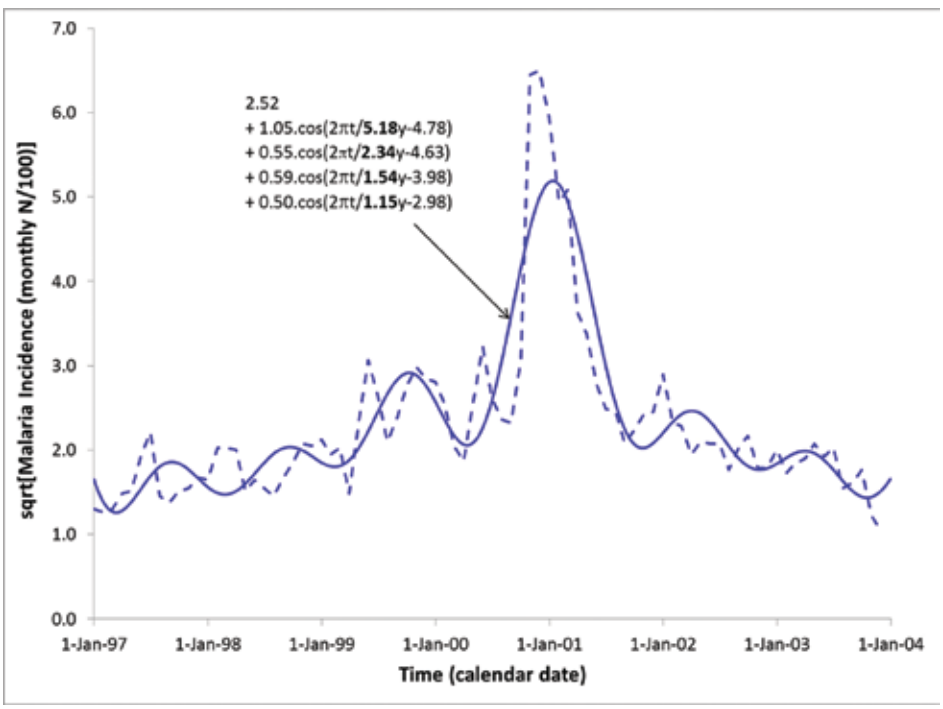

Figure 1. In view of a large outbreak resulting in influential (outlying) values, the monthly data on malaria incidence in Burundi recorded between 1997 and 2004 are transformed by taking their square root prior to analysis by the extended (linear-nonlinear) cosinor. Components with periods of about 5.2, 2.3, 1.5, and 1.15 years identified by least squares spectra and validated nonlinearly are included in a model plotted with the data. © Halberg Chronobiology Center.

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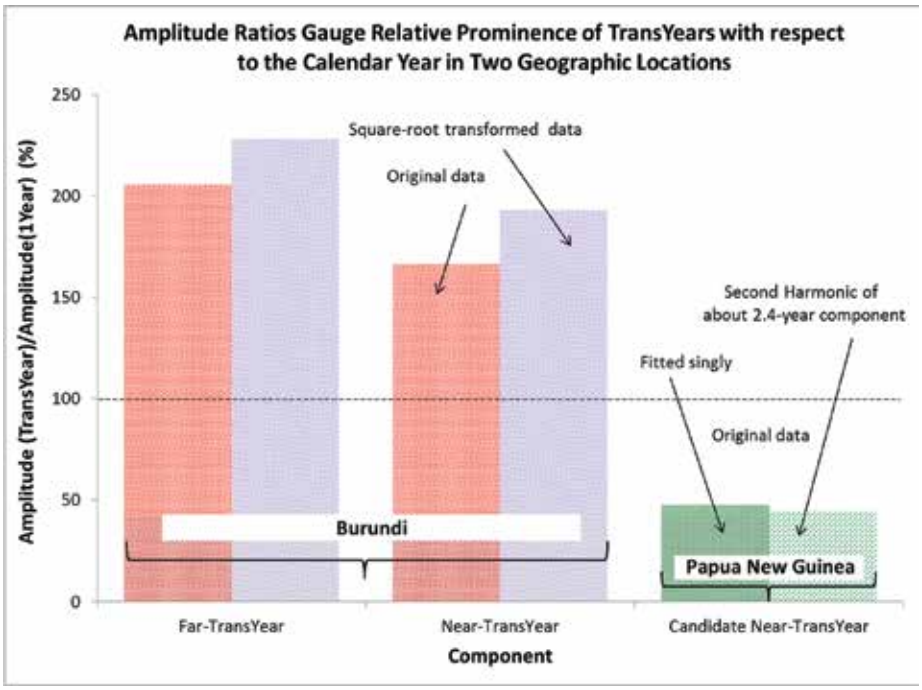
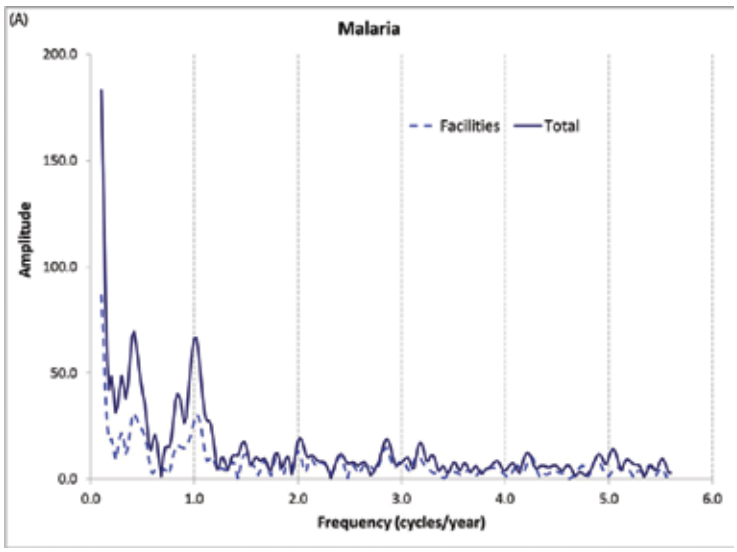


Figure 2. Amplitude ratios compare the relative prominence of the far- and near-transyear versus the calendar year in malaria incidence in Burundi (left and middle) and in Papua New Guinea (right). Despite the fact that both geographic sites are located near the equator, transyears are more prominent in Burundi but are only barely detected in Papua New Guinea. © Halberg Chronobiology Center.



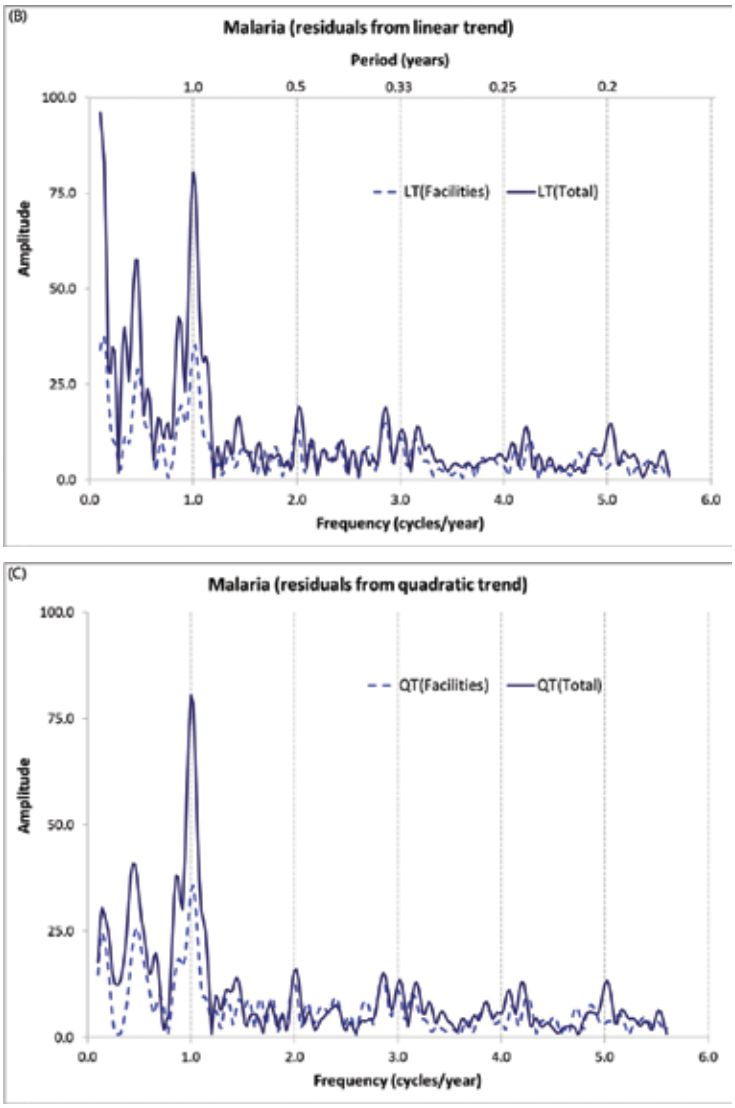


Figure 3. (A) Least squares spectrum of the monthly incidence of cerebral malaria in Papua New Guinea (original data). Spectral peaks correspond to a prominent 1-year component and a cycle with a period slightly longer than 2 years. The large amplitude of the low-frequency component may reflect the increasing trend seen in a plot of the data as a function of time (not shown). © Halberg Chronobiology Center. **(B)** Least squares spectrum of the monthly incidence of cerebral malaria in Papua New Guinea (residuals from a linear trend). In addition to the spectral peaks corresponding to the year and about 2.3-year components seen in the spectrum of the original data (**Figure 3(A)**), there is a smaller peak corresponding to a cycle with a period of about 1.15 years, which may also be the second harmonic of the about 2.3-year cycle. © Halberg Chronobiology Center. (**C)** Least squares spectrum of the monthly incidence of cerebral malaria in Papua New Guinea (residuals from a quadratic trend). Spectral peaks corresponding to the yearly and about 2.3-yearly components remain visible, while the low-frequency amplitude is considerably reduced by comparison to spectra of the original data and of residuals from a linear trend. With a series spanning no more than 10 years, it is not possible to distinguish between a quadratic trend and a cycle with a period of about 10 years or longer. © Halberg Chronobiology Center.

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	Facilities						Total					
	Period	[95%	CI]	Amplitude	[95%	CI]	Period	[95%	CI]	Amplitude	[95%	CI]
	Trial period = 2 years											
Original	2.392	2.084	2.700	30.93	-1.07	62.94	2.420	2.127	2.714	70.05	4.10	135.99
+LinTrend	2.147	1.961	2.332	29.30	7.67	50.93	2.229	2.019	2.440	58.76	14.02	103.50
+QuadTrend	2.104	1.900	2.308	26.24	4.56	47.91	2.228	1.977	2.478	42.14	3.87	80.40
	Trial periods = 10, 2 and 1 year(s)											
Original	15.346	8.607	22.085	93.50	63.98	123.02	17.629	8.924	26.333	247.54	118.83	376.26
	2.055	1.840	2.270	22.88	2.11	43.65	2.448	2.190	2.707	41.14	8.30	73.98
	0.985	0.952	1.017	33.80	13.14	54.46	0.992	0.970	1.013	77.53	45.86	109.19
+LinTrend	7.895	4.377	11.414	35.44	13.66	57.21	***11.879***	***1.871***	***21.887***	***116.30***	***-12.49***	***245.09***
	2.147	1.925	2.370	24.73	3.24	46.23	***2.338***	***2.079***	***2.596***	***41.20***	***7.44***	***74.96***
	0.986	0.953	1.019	33.43	12.55	54.32	***0.993***	***0.971***	***1.016***	***77.20***	***44.40***	***110.00***
+QuadTrend	6.160	2.488	9.831	24.81	0.15	49.47	5.161	3.597	6.725	35.28	0.88	69.69
	2.126	1.892	2.360	25.36	3.22	47.50	2.190	1.970	2.409	41.46	8.50	74.42
	0.987	0.954	1.021	34.12	12.43	55.82	0.996	0.975	1.018	80.29	47.96	112.63
	Trial periods = 10, 2 and 1(fixed) year(s)											
Original	15.614	8.684	22.544	94.12	63.97	124.28	21.895	5.081	38.708	309.80	0.33	619.26
	2.056	1.852	2.260	23.46	3.29	43.62	2.200	1.980	2.419	38.13	7.06	69.21
	1.000			32.42	12.40	52.44	1.000			77.49	46.83	108.16
+LinTrend	7.822	4.518	11.127	35.02	14.10	55.94	***11.405***	***2.988***	***19.821***	***106.28***	***15.08***	***197.47***
	2.137	1.926	2.347	25.13	4.27	45.99	***2.265***	***2.036***	***2.493***	***41.27***	***8.52***	***74.01***
	1.000			32.37	12.13	52.62	***1.000***			***77.00***	***45.43***	***108.56***
+QuadTrend	6.036	2.846	9.226	24.63	1.54	47.72	5.149	3.666	6.632	35.45	2.27	68.64
	2.117	1.898	2.337	25.81	4.30	47.33	2.185	1.977	2.393	41.61	9.82	73.40
	1.000			33.27	12.16	54.37	1.000			80.06	48.92	111.20
	Trial period = 1.2 years											
Original	1.189	1.031	1.346	15.84	-6.75	48.44	1.187	1.058	1.316	40.22	-27.36	107.81
+LinTrend	1.136	1.052	1.220	19.40	-3.05	41.84	1.154	1.074	1.235	43.06	-3.13	89.24
+QuadTrend	1.130	1.044	1.215	18.64	-3.52	40.80	**1.152**	**1.078**	**1.226**	**38.61**	**0.84**	**76.37**
	Trial period = 2.4(&2ndH) years											
Original	2.397	2.162	2.631	30.72	-6.26	67.70	2.405	2.197	2.612	69.37	-6.57	145.31
	1.198			15.39	-21.34	52.12	1.202			38.49	-37.30	114.28
+LinTrend	2.220	2.077	2.363	26.78	2.68	50.87	2.287	2.147	2.428	54.38	5.26	103.51
	1.110			15.93	-8.10	39.97	1.144			38.98	-10.19	88.16
+QuadTrend	1.982	1.925	2.040	23.21	3.03	43.40	***2.300***	***2.164***	***2.436***	***39.01***	***-1.81***	***79.84***
	0.991			35.91	15.58	56.24	***1.150***			***36.29***	***-3.61***	***76.20***
	Trial period = 10,2.4(&2ndH) years on residuals from 1-year fit											
Original	14.160	8.695	19.625	88.98	65.46	112.51	17.656	9.159	26.153	245.83	121.36	370.30
	2.357	2.110	2.603	19.79	-1.41	40.98	2.408	2.240	2.577	40.60	9.60	71.60
	1.178			7.90	-12.43	28.22	1.204			18.59	-11.26	48.45
+LinTrend	**8.008**	**4.596**	**11.421**	**35.59**	**15.31**	**55.87**	12.612	0.755	24.470	118.08	-28.72	364.87
	2.165	**2.048**	**2.283**	**23.80**	**3.95**	**43.66**	2.204	2.095	2.314	38.99	8.10	69.87
	1.083			**16.21**	**-2.85**	**35.27**	1.102			28.80	-0.59	58.18
+QuadTrend	6.042	3.160	8.923	24.49	3.33	45.65	5.133	3.829	6.437	35.08	4.44	65.71
	2.160	2.042	2.278	24.38	4.03	44.73	2.195	2.097	2.293	39.90	10.60	69.21
	1.080			16.94	-2.79	36.67	1.097			30.62	2.22	59.03

Table 2. Nonlinear results from several models fitted to monthly incidence of malaria in Papua New Guinea.

Nonlinear results from several models fitted to the data in Papua New Guinea are summarized in **Table 2**. A 1.0-year synchronized component is most prominent, whereas transyears are not detected with statistical significance (**Figure 2**, right). As seen from **Table 2**, the period of the circannual component has a CI overlapping the exact 1.0 (calendar) year. Accordingly, its period can be fixed in composite models. The period of the about 2.3-year component assumes consistent estimates irrespective of the model considered. As expected from the brevity of the series spanning only 10 years, this is not the case for the decadal component. Indeed, the low-frequency (about 10-year?) component is somewhat problematic: analyses of the original data tend to estimate its period between 17 and 22 years, shortened to about 11 years in some models including a linear trend, or toward 5 years when considering a quadratic trend. Nevertheless, for the total number of cases, the period estimate converges toward 11.4 years when a linear trend is included in the model, albeit with a very wide CI. A near-transyear with a period of about 1.15 years is also detected when fitted as a single

component or as the second harmonic of the about 2.3-year cycle, but its amplitude is much smaller than that of the yearly rhythm and it cannot be detected with statistical significance as part of a composite model including the yearly and decadal cycles. Only in the case of facilities when a linear trend is included in the model and analyses are carried out on residuals from the yearly component is the near-transyear detected with borderline statistical significance, the decadal cycle having an estimated period of 8.0 [CI: 4.6, 11.4] years.

As a compromise, a model consisting of a linear trend, a fixed 1-year component and about 10-year and 2.3-year components was used to approximate the data. For all cases (total), the period estimates were 11.4 [CI: 3.0, 19.8] and 2.26 [CI: 2.04, 2.49] years. Using these estimates, a composite model was fitted by the linear cosinor, including the candidate 1.1-year component. The model as a whole and all its components are detected with statistical significance for both facilities and all cases (P<0.001). Results from this multiple-component fit were used to approximate the data, as illustrated in **Figure 4**.

Whatever model is considered, malaria in Papua New Guinea is characterized by a prominent yearly component, a cycle with a period slightly longer than 2 years and a trend that may correspond to a longer cycle, possibly with a period of about 10 years. No far-transyear is detected and a near-transyear may be the second harmonic of the about 2.3-year cycle, much less prominent than the calendar year, in sharp contrast with results in Burundi, also located near the equator. Thus the incidence of malaria differed not only by the absence in Burundi of a calendar-year component found in Papua New Guinea, but also by the presence of prominent far- and a near-transyears in Burundi, but only a candidate near-transyear in cerebral malaria incidence in Papua New Guinea. Accordingly, the near-transyear-to-calendar year amplitude

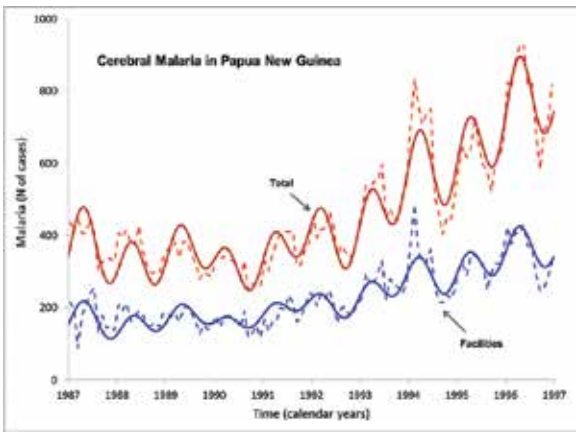

Figure 4. Monthly data on the incidence of cerebral malaria in Papua New Guinea are fitted with a model consisting of a linear trend, a 1.0-year synchronized rhythm, about 11.4-year and about 2.3-year components, and a candidate 1.1-year near-transyear. Whereas the contribution of each component to the composite model can be seen by the naked eye, this is not the case for the near-transyear that only has a very small amplitude. © Halberg Chronobiology Center.

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ratio, if the presence of a near-transyear in Papua New Guinea is accepted, was smaller than 100% (**Figure 2** (right)).

Variable	Period	[95% CI]		Amplitude	[95% CI]		[1-parameter limits]	
API	17.21	12.97	21.45	0.98	0.00	1.95	0.51	1.45
	9.60	7.64	11.56	0.66	-0.24	1.56	0.23	1.09
SPR%	18.42	13.70	23.13	1.02	-0.07	2.12	0.50	1.55
	10.07	8.02	12.12	0.73	-0.28	1.73	0.24	1.21
SfR	16.62	11.36	21.88	0.13	-0.03	0.30	0.05	0.21
	8.27	7.24	9.30	0.15	-0.01	0.32	0.07	0.23
Pf%	25.71	18.97	32.44	4.84	2.11	7.57	3.53	6.15
	9.15	8.14	10.16	3.18	0.49	5.87	1.89	4.48
Pf%	22.70	13.29	32.11	5.50	0.40	10.60	3.35	7.66
	15.94	11.04	20.84	3.71	-1.57	8.99	1.48	5.94
	8.81	8.02	9.60	2.80	0.91	4.69	2.00	3.60

All models fitted with a linear trend.

API: Annual parasite incidence (malaria positives in 1000 population).

SPR%: Slide positivity rate (positives per 100 slides examined).

SfR: Slide falciparum rate (Pf infection per 100 slides examined).

Pf%: Pf infections per 100 malaria positives.

Table 3. Nonlinear results of yearly incidence of malaria in Thailand.

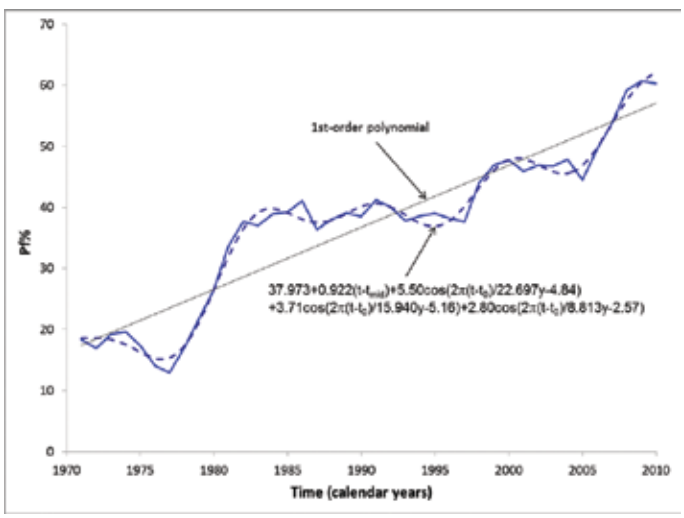

Figure 5. The yearly data on the percentage of *Plasmodium falciparum* infections (expressed per 100 malaria positives) in Thailand are fitted with a model consisting of a linear trend and cycles with periods of about 22.7, 15.9, and 8.8 years. © Halberg Chronobiology Center.

Tentative decadal components were also found by linear-nonlinear cosinor in the yearly data from Thailand (**Table 3**). Results for the percentage of *P. falciparum* infections per 100 malaria positives are illustrated in **Figure 5**.

4. Discussion

Malaria is transmitted in tropical and subtropical areas where Anopheles mosquitoes can survive and multiply. Malaria parasites can complete their growth cycle in the mosquitoes. Temperature is particularly critical. Generally, in warmer regions closer to the equator, transmission is more intense, and malaria can be transmitted year-round. It is not surprising then that components with periods other than 1 year may also be detected, reflecting the influence of solar and other factors not directly related to temperature.

Our interest in the relative contributions of the seasons vs. helio-, interplanetary and terrestrial magnetism was stimulated by the report by Dimitrov et al. [8] of a sharp calendar-yearly peak in the spectrum of monthly admissions of cerebral malaria in Papua New Guinea (latitude -6°; 1987–1996) with no comparable transyears (components longer than a year with a CI of their period not overlapping the calendar year). Decadal and longer cycles cannot be examined in the short series of malaria incidence in Burundi analysed herein, although, as noted, an about 5-year cycle can be the second harmonic of the Horrebow-Schwabe cycle. An about 2-year component is also found in interplanetary and geomagnetism as well as in the El Niño Southern Oscillation (ENSO) and may show cross-wavelet coherence with malaria in certain regions of Thailand [9].

Figure 6. An about 5.6-year component was also detected in yearly data from Chizhevsky on mortality from cholera in Russia [19, 20]. © Halberg Chronobiology Center.

An about 5-year component in his time series on cholera incidence was communicated to Alexander Leonidovich Chizhevsky [17] by Vladimir Boleslavovich Shostakovich (**Figure 6**) [18]. Decadal and multidecadal signatures are found in diphtheria, croup, relapsing fever, and

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cholera at a time when these diseases were rampant in meta-analyses [18] of statistics assembled descriptively by Chizhevsky [17]. Chizhevsky also reports on data from Dr. SI Ivanchenko regarding the incidence of malaria in the North Caucasus from 1916 to 1930. He noted an inverse relationship between the incidence of malaria and air ionization. Albeit short, the record can be characterized by a decadal component, as illustrated in **Figure 7**.

Figure 7. A decadal component characterizes the incidence of malaria in Russia between 1916 and 1930, as seen from the low-frequency spectral peak, resolved nonlinearly as an about 12.6-year component, which is fitted to the data (top right). Note a secondary smaller spectral peak corresponding to a period of about 2.3 years, as observed also in the data from Burundi (**Figure 1**) and Papua New Guinea (**Figure 3**). © Halberg Chronobiology Center.

In the case of malaria, it is surprising to see the presence of a calendar yearly component in the tropical region of Papua New Guinea, which is absent in Karuzi, Burundi (at 3° from the equator at a latitude, but not a longitude similar to that of Papua New Guinea). The original publication on malaria in Burundi [10] used data on rain and temperature and a vegetation index to predict malaria incidence. The terrestrial environmental variables could also be related to solar activity, as reviewed by Clayton [19] and Abbot [20].

Both the near- and far-transyears are features of the solar wind's speed and of geomagnetics. Space weather and geomagnetism may act via rainfall and its consequences. Just as helio-, interplanetary, or geomagnetism can influence sudden cardiac death [2], they may also influence communicable diseases, probably via the host, whose steroidal defence shows a decadal cycle [21] and/or by the invading microorganism. Bacterial mutations can also undergo a cycle mirroring that of sunspots [22–24].

Funding support:

Funding for this study is from Halberg Chronobiology Fund (GC), University of Minnesota Supercomputing Institute (GC). Processing charges are funded by the Academic Unit of Primary Care and Populations Sciences, University of Southampton, UK.

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Chapter 3

Malaria in Pregnancy

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64611

Abstract

Malaria infection during pregnancy is an important public health problem with substantial risks to both the mother and foetus. Pregnant women are the most vulnerable group of malaria-associated morbidity and mortality. A pregnant woman has an increased risk (up to four times) of getting malaria and twice the chances of dying from malaria, compared to a non-pregnant adult, becuase the immune system is partially suppressed during pregnancy. Malaria in pregnancy not only affects the mother but also has a dangerous sequel for the developing foetus, resulting in premature delivery or intrauterine growth retardation. Diagnosis of malaria in pregnancy remains a challenge due to the low parasite density and placental sequestration of *Plasmodium falciparum*. Thus, there is an urgent need for new diagnostic methods to detect malarial parasites in the pregnant women. Though antimalarial drugs are available, which can be safely given in the pregnancy, increasing drug resistance of malarial parasite may pose a big problem in the future. In this chapter, we review the burden of pregnancy-associated malaria (PAM), its pathogenesis, diagnostic issues during pregnancy and recent guidelines for chemoprophylaxsis and treatment.

Keywords: malaria, pregnancy

1. Introduction

Pregnancy-associated malaria (PAM) is a major health problem, which not only affects mother but also affects the developing foetus. Worldwide, 50 million women are at risk of PAM per annum [1, 2]. Depending upon the transmission pattern of malaria, PAM has been studied in two different geographical regions: one with a high and stable transmission rate (Africa) and another one with a low and unstable transmission rate (Asia-Pacific region). The majority of

the studies are from Sub-Saharan Africa, where *Plasmodium falciparum* causes the majority of the cases. Approximately 25 million pregnant women are at risk of *P. falciparum* infection, and 25% women had evidence of placental infection at the time of delivery [3]. Due to the high endemicity of malaria in these regions, adults develop natural immunity due to repeated infections; thus, PAM rarely results in fever and remains undetected and untreated. On the other hand, Asia-Pacific region has a low transmission of *P. falciparum* and women have little acquired immunity against malaria. In Asia-Pacific region, most pregnant women are at a risk of *Plasmodium vivax* infection. Asia-Pacific region consists of WHO South-east Asia and Western Pacific regions [3]. Approximately, 75 million women became pregnant in the Asia-Pacific region in 2007 [4]. Majority of these cases were from India and China. In low transmission settings, malaria in pregnancy is associated with anaemia, an increased risk of severe malaria and it may lead to spontaneous abortion, still birth, prematurity and low birth weight. In such settings, all pregnant women are affected irrespective of the number of times they have been pregnant. Infection with *P. vivax*, as well as with *P. falciparum*, leads to chronic anaemia and placental infection, resulting in birth weight reduction and increased neonatal mortality. However, reduction in birth weight in *P. vivax* is only two-third as associated with *P. falciparum*. Effect of *P. vivax* appears to increase with successive pregnancies [5].

One should be cautious while accessing the burden of malaria as there are many gap holes in the knowledge of epidemiology and burden of malaria in pregnancy. Long-term prospective studies are required for accessing the true incidence of PAM. Study designs and methodology should also be kept in mind while calculating the estimates of the burden of malaria [6–8]. Recently, verbal autopsy has been used in a study published in *The Lancet* [7], in which authors have interviewed the relatives of a person who has recently died to determine a cause of death. The results are astonishing in terms of numbers as there is a great disparity between malaria death rates of Lancet group estimates and WHO estimates [8]. The Lancet study has projected the estimates of 1.24 million deaths worldwide which includes 7,14,000 deaths under the age of 5 as compared to WHO estimates of 6,55,000 deaths worldwide which includes 5,63,300 deaths under the age of 5 [8]. The reliability of these findings is a matter of debate, and there is an urgent need to review these findings involving a broader group of experts, so that malaria control programs can be reformulated with newer targets. In Africa with stable transmission, the median prevalence of maternal malaria infection (defined as peripheral or placental infection) in all gravidae has been estimated to be 27.8% [1, 9]. These are the minimum estimates as they are based on single-point prevalence and are based upon light microscopy, which cannot detect the sub-microscopic parasitemia of pregnancy. So, more studies are required which can detect malaria with high sensitivity and specificity such as *Plasmodium* DNA detection by polymerase chain reaction (PCR) and placental histology. In a low-transmission African setting, the median prevalence of peripheral and placental parasitemia has been estimated to be 13.7 and 6.7% respectively [10]. However, outside Africa regions with low-transmission have respective estimates of 6.2 and 9.6% [10]. It has been estimated that placental infection in regions of stable transmission in Africa are identified more frequently in dry seasons as infection acquired during rainy season may persist for several months in placenta [11, 12]. Thus, during the low-transmission season also one can get the PAM with a higher frequency.

Although *P. vivax* has been known to be associated with malaria in pregnancy for the quite long time, its impact has been accessed only recently [13]. Studies from India, Thailand and Papua, Indonesia showed that low birth weight neonates and anaemia are more pronounced as compared to non-infected pregnant women, but severity was less as compared to *P. falciparum* associated malaria in pregnancy [14–16].

2. Risk factors

2.1. Gravidity

Primigravidae are at increased risk of infection in high-transmission areas, whereas they are less marked in low-transmission areas as women of all gravidities are susceptible to severe maternal and foetal outcome. Thus, parity specific immunity plays an important role in PAM.

2.2. Maternal age

Younger age group is an independent risk factor for PAM. Thus, age-specific immunity protects an individual from PAM.

2.3. HIV infection

HIV increases the risk of acquiring malaria in pregnancy by weakening the immunity. It also hampers the cytokine responses to malaria. These patients have decreased IL-12 levels, which in turn lead to decreased interferon-γ leading to increased susceptibility to placental malaria in HIV. Malaria increases HIV viral load in pregnancy and also increases the expression of CCR5, which is an important co-receptor for HIV cell entry. Malaria and HIV are linked in other ways also, that is, *The DARC gene* which protects against *vivax* malaria might increase susceptibility to HIV [8, 17].

2.4. Effect of *Plasmodium* species

All the four species of Plasmodium can infect pregnant women, but the prevalence and effect of *Plasmodium ovale* and *Plasmodium malariae* in pregnancy are currently unknown. *Plasmodium knowlesi* is the commonest cause of malaria in Malaysia, but it is relatively rare during pregnancy [18]. Pregnant women are at increased risk to both *P. vivax* as well as *P. falciparum* infection, but the risk is more for *P. falciparum* due to placental cytoadherence. Mixed infections can also occur in pregnancy, but the prevalence of mixed infections decreases with increasing age and gravidity. Differences in between *P. vivax* and *P. falciparum* are important for understanding pathogenesis of malaria in pregnancy due to different species. *P. vivax* infects reticulocytes after binding of merozoites with Duffy antigen receptor, which is lacking in African population leading to protection against *P. vivax* [15]. This also helps in limiting the parasitemia in *P. vivax* infections as compared to *P. falciparum* infections where mature RBCs are invaded leading to relatively high parasitemia [11]. Infected RBCs with *P. vivax* do not express erythrocyte surface proteins required for sequestration, which is well documented in

P. falciparum infections [11, 19]. This heavy sequestration in placenta is discussed below that is associated with high parasitemia in placenta leading to low birth weight neonates and anaemia. Lastly, relapsing hypnozoite forms characteristics of *P. vivax* may complicate the clinical course of infection. This suggests that systemic infection in mother is playing an important role in pathogenesis of PAM due to *P. vivax* as compared from *P. falciparum* where placenta is heavily infected by malaria parasite.

3. Pathogenesis

3.1. Placental changes associated with malaria

Infected erythrocytes tend to lodge in much higher densities in intervillous space as compared to the peripheral circulation [20]. Due to sequestration of infected erythrocytes, trophozoites and shizonts are absent in peripheral blood [21]. PAM is also associated with increased numbers of phagocytic cells in the intervillous space along with the deposition of malarial pigment (haemozoin). Precise and accurate diagnosis requires an examination of histological sections of placenta. Based upon parasitized erythrocytes and haemozoin, placental changes have been categorized into four classes (**Table 1**).

S. no.	Category	Description
1.	Not infected	No evidence of parasitized erythrocytes or malarial pigment
2.	Active-acute	Parasitized erythrocytes present, with no or minimal pigment deposition in fibrin
3.	Active-chronic	Presence of parasitized erythrocytes along with substantial amount of pigment in cells or in fibrin
4.	Past	Absence of parasites but presence of pigment

Table 1. Description of placental pathology in PAM (adapted from Ismail et al. [67]).

Recently, studies have shown that chronic infections are associated with lower maternal haemoglobin and low birth weight due to foetal growth retardation, whereas preterm births are more closely associated with acute infection having high parasitemia.

3.2. PAM is not always associated with pathology

It has been seen that microscopic examination of blood smears is not a sufficient diagnostic tool to detect the sub-microscopic infections. These are mainly detected by polymerase chain reaction, and a study by Mankhambo et al. [22] has shown that sub-microscopic infections are not associated with low birth weight or with lower maternal haemoglobin. Thus, it is the density of the parasites which matters a lot and women might not suffer from adverse effect of PAM, in whom density of the parasites is under control. Therefore, PAM is common but is not always associated with adverse outcomes.

http://dx.doi.org/10.5772/64611

3.3. Plasmodium infected erythrocytes that cause malaria in pregnant versus non-pregnant women

Placental-infected erythrocytes with *P. falciparum* differ in a number of ways as compared to infected erythrocytes from non-pregnant women (**Figure 1**) [19]. Placental-infected erythrocytes adhere to glycosaminoglycan receptors throughout the intervillous space [23, 24]. Chondroitin sulphate A has been considered to be the most predominant receptor placental adhesion receptor, which is present as a glycosaminoglycan side chain [25] of tissue thrombomodulin [26] and also as a part of secreted low-sulphated aggrecan in intervillous space. In contrast, infected erythrocytes of non-pregnant women do not use this receptor, and sequestration occurs near to vascular wall in other tissues. Another important difference is that rosettes formation does not occur among placental infected erythrocytes, which is a common feature of infected erythrocytes of non-pregnant women [27, 28]. Placental erythrocytes can adsorb IgM [29], and the adhesion ligands vary in their sensitivity to trypsin digestion [30]. On the other hand, infected erythrocytes of non-pregnant women can adsorb IgM on their surface. Thus, parasite antigens causing adhesion of infected erythrocytes to chondroitin sulphate A are different from those antigens that are expressed on infected erythrocytes during non-placental *P. falciparum* infections. All these surface expressed antigens are collectively known as variant surface antigens (VSAs).

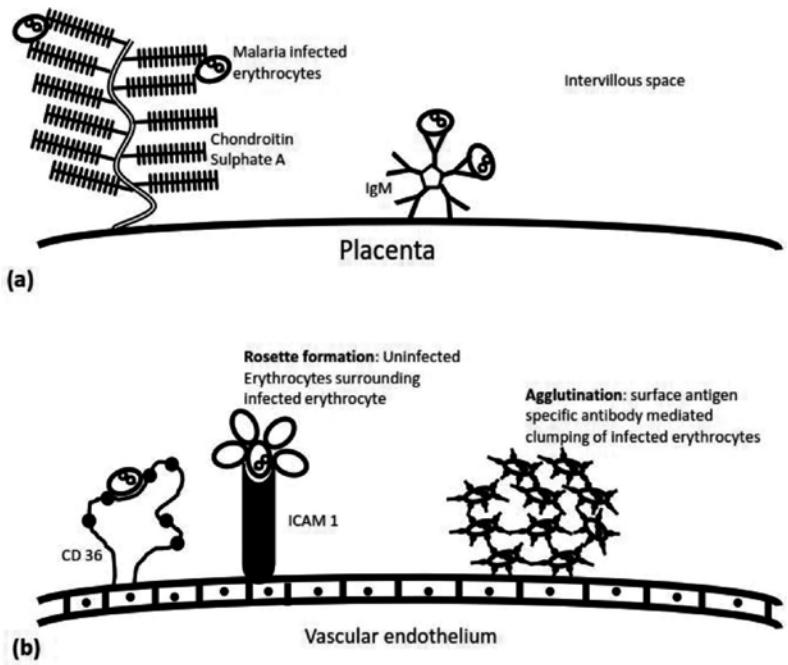

Figure 1. Comparison of malaria-infected erythrocytes in pregnant women (a) versus non-pregnant individuals (b). In pregnant women, infected erythrocytes adhere to chondroitin sulphate A receptor present in intervillous area of placenta, whereas in non-pregnant women infected erythrocytes adhere to CD36 or ICAM1 and also exhibit rosette formation and agglutination [19].

3.4. Humoral immune response to variant surface antigens in pregnancy

VSAs are the main targets of IgG which provides the protective immunity in response to repeated episodes of *P. falciparum* infections in non-pregnant individuals. Thus, if VSA is different among pregnant and non-pregnant individuals, then susceptibility to infection with *P. falciparum* in previously clinically immune women when they become pregnant could easily be explained. This would also explain the increased chances of PAM in women of low gravidity in areas with high transmission. A study by Fried et al. [31] has provided an evidence which shows that VSA expressed by placental infected erythrocytes are immunologically distinct. They have shown that IgG derived from multigravidae who were exposed to *P. falciparum*, inhibit the adhesion of infected erythrocytes to chondroitin sulphate A that are obtained from primigravidae [32]. Whereas, adhesion of infected erythrocytes to chondroitin sulphate A is not inhibited by serum from either primigravidae or men. IgG1 is known to be the predominant subclass of IgG against VSA in PAM [33, 34].

3.5. Humoral immune response to other antigens in pregnancy

Humoral immune response to other blood stage antigens have also been known to provide immunity in PAM. Thus, young age is an independent risk factor for PAM. The relative protection from severe malaria has also been observed in the area of high exposure to malaria prior to pregnancy. However, humoral immune response to pre-erythrocytic antigens/blood stage antigens other than VSA is not sufficient to provide adequate immunity in PAM [32].

3.6. Protective antibodies in PAM

There is an inverse relationship between gravidity and PAM. Protective levels of antibodies against VSA inhibiting adhesion to chondroitin sulphate A have an inverse relationship to low birth weight, preterm delivery and low maternal haemoglobin levels [35, 36]. More studies are required to identify the other targets of protective immune response in pregnancy.

3.7. Increase in monocyte infiltrates in placental intervillous space

Maternal mononuclear cells are stimulated by sequestrated infected erythrocytes in placenta. Activated mononuclear cells secrete β-chemokines that are chemotactic for macrophages and monocytes. Macrophage migration-inhibitory protein (MIF) helps in retention and activation of macrophages [37, 38]. Thus, induction of these cytokines helps in increase of monocytes and macrophages in the intervillous space in PAM. These cells also act as antigen presenting cells to T cells [39].

3.8. Placental cytokines in PAM

During normal pregnancy, cytokine balance is shifted towards Th2-type immune response [40], and Th1-type immune response is associated with adverse maternal and foetal outcomes such as maternal anaemia, spontaneous abortions and premature deliveries. The Th1-type of immune response aids in clearing the parasites from the placenta. The various mechanisms documented include increase in phagocytic activity of macrophages and increase in nitric

oxide activity. In addition to that, oxygen-free radicals along with stimulation and proliferation of T-cells also play an important role in clearing the parasites. Thus, Th1-type immune response helps in fighting against the malaria infection, but its overproduction is harmful as it can terminate the pregnancy. The ill effects of pro-inflammatory cytokines are counterbalanced by IL-10 and have been shown to be up-regulated in intervillous space. Thus, it is the delicate balance between Th1 and Th2 immune response which provides immunity in PAM.

3.9. Role of innate cells in PAM

Macrophages, dendritic cells, natural killer (NK) cells, γδ T cells are the arms of innate immune response, which helps in shaping the adaptive immune response to malaria. They help in early production of cytokines which determine the response to infection by modulating the immune response. Placental macrophages help in eliminating the parasites. Infected erythrocytes also adhere to NK cells to produce interferon-γ. Adherence to dendritic cells is mediated by CD36 which further helps in antigen presentation to T cells [41, 42]. NK T cells have been shown to be protective against blood-stage malaria infections in athymic mice. Toll-like receptors (TLRs) help in recognition of asexual-stage of *P. falciparum* by innate cells. Polymorphism in TLR 4 and TLR 9 is known to be associated with low birth weight, foetal growth retardation and maternal anaemia [43, 44]. There are few studies available which confirm the specific roles of innate immune response in PAM, so more studies are required to unveil the role of innate immune response in PAM among humans.

3.10. T and B cell response in PAM

During pregnancy, very delicate immunological balance is maintained so that mother's immune system must remain tolerant to the developing foetus but provide protection against invading pathogens on another side. Earlier studies have shown that pregnancy predisposes the women to severe disease due to the suppression of cell-mediated immune response [45]. However, these days submicroscopic parasitemia is well documented in which slides for peripheral smear are negative, but PCR is positive. It has been postulated that decrease in T-cell proliferative responses might be caused by the absence of memory T cells due to sequestration as compared to earlier concept of immunosupression [46].

Fievet et al. [47] investigated the immune response to *P. falciparum* antigen in Cameroonian primigravidae, evolution after delivery and during the second pregnancy. They have shown that levels of antibodies to Pf155/RESA are lower during the pregnancy in comparison to levels of antibodies after the delivery. However, levels of *P. falciparum* asexual blood stages are higher during the pregnancy than after delivery. This increase during pregnancy has remained unexplained and has not been reported earlier. It has been proposed that due haemodilution and passive transfer of IgG to developing foetus, there occurs a decrease in maternal IgG levels during pregnancy. It has also been postulated that immunosuppressionwhich occurs during pregnancy may impair the T-cell subsets differentially leading to impairment of IL-2 cellular response,but proliferative responses, IL-4, IFN-γ responses are either not affected or moderately increased. This suppression of IL-2 has been linked to the hypothesis supporting the transient depression of Th1 immune response during pregnancy. However, the suppression

of IL-2 is not parallel with the reduction of IFN-γ, suggesting Th1 subset might be differential affected by the PAM [48, 49]. Fievet et al. [49] have shown that cellular immune response is related to previous placental infection and parity even after the pregnancy in relation to *P. falciparum* infections. They have shown that T-cell proliferative responses and cytokine production (IL-2, IL-4, IL-10 and IFN-γ) is significantly higher in multigravidae as compared to primigravidae when cells are stimulated with chondroitin sulphate A-adhering RP5 strains of *P. falciparum* but not with other strains which lack adherence to chondroitin sulphate A. These findings will be useful in designing the vaccine for malaria in pregnancy, if parity-dependent stimulation of cytokine production is important in PAM. In PAM, high levels of IgGspecific against VSA has been documented along with higher number of VSA-specific memory B-cells. Surface-exposed epitopes of VAR2CSA are the main target of antibody production [49].

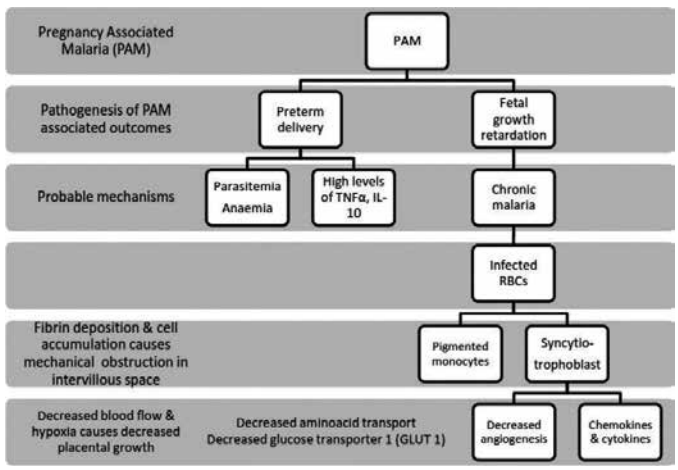

Figure 2. Probable mechanisms by which pregnancy-associated malaria can cause preterm delivery or foetal growth retardation [19].

3.11. Pathogenesis of foetal growth retardation in malaria

Foetal growth retardation is known to be associated with chronic malaria. Whether it occurs due to mainly due to events occurring near the delivery (e.g. cytokines, impaired uteroplacental blood flow or biochemical imbalance in the placenta) or due to chronic infection which slowly compromise the foetal growth, are still unknown. Malaria is known to impair uterine spiral arteries remodelling during trophoblast invasion, leading to decreased placental circulation [50]. Mechanical obstruction of intervillous space by infected erythrocytes, monocytes and deposition of fibrin are also known to compromise the placental circulation [51]. Cytokines released by monocytes could directly or indirectly affect nutrient transport mechanisms. Thus, decreased blood flow, impaired placental development and impaired nutrient transport are the probable mechanisms by which foetal growth retardation occurs (**Figure 2**).

3.12. Pathogenesis of preterm delivery due to malaria

Preterm birth occurs delivery before 37 weeks of gestation, and it is known to be closely associated with high parasitemia, maternal anaemia, increased levels IL-10 and TNF-α [38]. But how it occurs is still unknown.

3.13. Pathogenesis of anaemia in malaria

Anaemia in malaria is a multifactorial, caused by a combination of erythrocyte destruction (both infected and uninfected erythrocytes) and bone marrow dysfunction. Other associated factors such as hookworm infection, HIV infection, chronic inflammation, iron and folic acid deficiency, accumulation of pigmented monocytes and their inflammatory mediators are known to suppress the erythropoesis [52, 53].

4. Role of hormones in PAM

Malaria in pregnancy has been associated with an increase in cortisol levels, which may reflect a stress response to malaria. It has also been postulated to suppress the immune system, but exact mechanism how it occurs is still a matter of debate. In late pregnancy, it is also associated with reduced oestradiol levels [54, 55].

5. Maternal outcomes in malaria

The maternal outcome in malaria depends upon the degree of immunity acquired by the pregnant women. Thus, epidemiological transmission has an important effect on maternal outcome. In areas of high transmission, only few individuals get the clinical signs and symptoms of malaria and same applies to pregnant women [10]. In Africa, malaria is responsible for approximately 26% of severe anaemia cases among pregnant women of all gravidities [2]. Hospital-based studies have shown that malaria-related maternal mortality rate among pregnant women, varies from 0.5 to 23.0%, whereas it varies from 2.9 to 17.6% among community-based studies [56]. However, in areas having low, unstable and seasonal transmission, women are more susceptible to develop the severe disease. In India, malaria-related maternal mortality rate among pregnant women varies from 7.0 to 66.6%, and in Thailand it varies from 10 to 27% [57]. Targeted treatment approach is required to curtail these figures of mortality.

6. Child outcomes in malaria

Malaria has the devastating effects on the developing foetus resulting in various outcomes (**Figure 3**). Low birth weight is an independent risk factor for the increase in infant mortality

rate [12, 58]. Risk of low birth weight doubles in primigravidae having placental malaria. Malaria in pregnancy is responsible for 20% of low birth weight deliveries in Sub-Saharan Africa [9]. In areas of high transmission, women have good-level immunity acquired due to repeated infections, thus decreasing the episodes of febrile illness, an important predisposing factor which causes preterm delivery. Furthermore, it is the intrauterine growth retardation which is the major cause of low birth weight babies as compared to areas having low rates of unstable transmission where preterm delivery is likely to be a more important cause of low birth weight due to poor levels of acquired immunity [10]. Malaria in pregnancy is also responsible for increased neonatal deaths, stillbirths, spontaneous abortions and anaemia. Steketee et al. [1] have reviewed the similar factors in Sub-Saharan Africa and estimated low birth weight, preterm delivery and stillbirth attributable to malaria in pregnancy, to vary from 8 to 14%, 8 to 36% and 13 to 70% respectively.

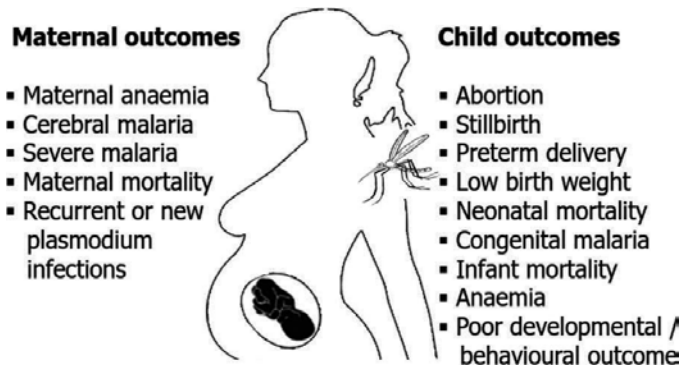

Figure 3. Maternal and child outcomes associated with malaria in pregnancy [10].

Placental malaria is also known to reduce the maternal antibodies to the foetus resulting in other infections such as measles and *Streptococcus pneumonia* [59, 60]. Recent studies have reported a rise in congenital malaria whose prevalence varies from 8 to 33% [61–63]. The increase may attributed to increase in drug resistance, HIV, increase in virulence and increased detection rates. Data is lacking which can provide direct evidence of malaria in pregnancy affecting development of the child in the long term. However, study from Malawi [64] has shown that placental malaria is an independent risk factor for anthropometric status in infancy. Other risk factors such low birth weight, anaemia can hamper the development of the brain and may result in cognitive disorders.

7. Diagnosis of PAM

Diagnosis of malaria in pregnancy is challenging as *P. falciparum* parasites are either absent or undetectable in peripheral blood, due to the sequestration of parasites in placenta [65]. This is due to the fact that infected erythrocytes are sequestered in the intervillous space [23]. Infections having a low parasite density may also affect the pregnant women and her devel-

oping foetus [66]. Thus, early diagnosis is essential for the timely initiation of treatment. Although, it is considered to be a gold standard for histological examination of the placental malaria, it is not applicable for routine diagnosis [67, 68]. However, placental examination is not possible before the delivery and thus, antenatal placental infection can only be inferred by peripheral blood smear examination.Although polymerase chain reaction (PCR) has the potential to diagnose malaria in pregnancy with higher sensitivity and specificity. A recent study by Mayor et al. [69] have shown that among the PCR positive for *P. falciparum* in peripheral and/or placental blood samples, 71.3, 61.5, 60.7 and 58.2% were negative by peripheral microscopy, by HRP2 ELISA in plasma, by HRP2 RDT in plasma and by histology respectively. However, molecular techniques are costly and are not available everywhere. Studies [70, 71] have shown a marked underestimation of malaria infection in pregnant women when diagnosed by standard microscopy in peripheral and placental blood as compared to molecular techniques such as polymerase chain reaction (PCR). Thus, there is an urgent need for more accurate diagnostic tools to detect malaria in pregnancy to prevent the negative clinical impact of hidden infection in pregnancy.

8. Prevention and management of PAM

Prevention of PAM is an important public health issue. Mainly, three-pronged approach has been advised for reducing the burden of PAM which are as follows [72]:

1. Effective case management
2. ITNs: use of insecticide-treated nets (ITNs)
3. IPT: intermittent preventive therapy in areas of stable transmission

In areas with low or unstable transmission of malaria, PAM can be controlled by timely diagnosis, and treatment of acute cases as IPT will be not very useful as it is in high transmission areas. Malaria in non-immune pregnant women are prone to severe disease, thus early initiation of antimalarials along with supportive management is very useful. Use of ITNs is useful in high transmission areas as its use will decrease the exposure to infective mosquito bites, thus decreasing the risk of placental malaria, low birth weight, still births and furthermore, continue to protect the newborn baby during infancy, since most babies sleep with their mothers [73]. However little is known about their effect in decreasing the clinical malaria and low birth weight in low transmission areas [74].

PAM in high transmission areas may be associated with placental parasitemia, causing maternal anaemia even in the absence of documented peripheral parasitemia. Therefore, it is recommended to treat presumptively whenever pregnant women present with severe anaemia, whether or not she has a history of fever and whether or not peripheral parasitemia is present [75, 76]. It is also recommended to give iron and folic acid supplementation to all pregnant women as a part of routine antenatal care. A study from Western Kenya has shown a dramatic reduction of 38% in the incidence of malaria parasitemia, 47%

reduction in anaemia and 28% reduction in the prevalence of low birth weight babies in those pregnant women who regularly used ITNs [77].

IPT has replaced the previous policy of chloroquine chemoprophylaxsis given weekly [78], as it was associated with poor patient compliance and increased chances of developing drug resistance [79]. Curative treatment dose of effective anti-malarial drug is given at predefined intervals during the course of pregnancy. As shown in **Figure 4**, all women should receive at least two doses of IPR in the second and third trimesters of pregnancy with the aim of clearing the placenta from parasites at the time of rapid foetal growth [80]. Though a single dose is also beneficial,yet one should aim for giving 2–3 doses. Sulphadoxine-pyrimethamine (SP) is the most common drug used in IPT, due to its long half-life and good safety profile in pregnancy [75, 81]. However, SP resistance in *P. falciparum* is prevalent throughout the Sub-Saharan Africa. The Queen Elizabeth Central Hospital Epidemiology of Resistance in Pregnancy-Associated Malaria (QuEERPAM) study [82] has shown promising results regarding the antenatal use SP and concluded that it does not exacerbate PAM despite the expansion of drug-resistance *P. falciparum*. Thus, in areas with substantial resistance, SP can be used as a component of antenatal care because pharmacologically, SP in the presence of partial host immunity can clear the resistant parasites, with limited placental inflammation. Thus, artemisinin-based combination therapy in IPT must now be evaluated along with priority-based research on evaluating the effectiveness and safety profile of newer antimalarial drugs [83, 84]. Recently, artemisinin-based chemotherapy has been found to be safe for the treatment of *P. falciparum*-associated malaria in pregnant females during first trimester. In near future, quinine-based antimalarial therapy during first trimester might be replaced by artemisinin combination therapy due to the encouraging results shown by Moore et al. [85].

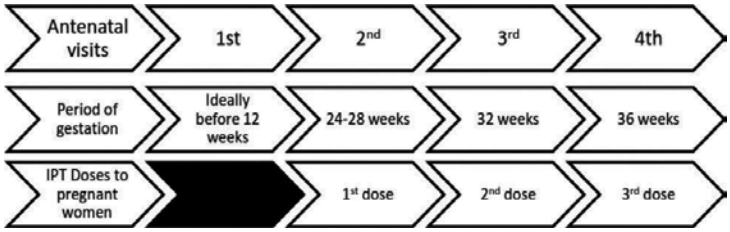

Figure 4. Intermittent preventive treatment (IPT) dosing schedule as recommended by World Health Organization. All women should receive at least two doses of IPT after quickening, as it helps in clearing the parasites at the time of rapid foetal growth. IPT may also be given at fourth visit, if not received the requisite number of doses. Maximum benefit is derived from 2 to 3 doses.

9. Conclusion

Malaria in pregnancy is an important public health problem. It not only affects mother but also hampers the growth of developing foetus. In areas with high transmission rate of ma-

laria, population is relatively immune and patients do not present with symptomatic malaria, which delay the diagnostic workup. Moreover, predominant *Plasmodium* species in such areas is *P. falciparum* that is known to cause sequestration of infected RBCs in placenta leading to compromise in normal physiology of placenta. In areas of low-transmission area, *P. vivax* is the predominant species causing malaria in pregnancy. It mainly causes systemic ill effects as compared to *P. falciparum* infection. Moreover, relapse of malaria could occur due to hypnozoites that could further complicate *P. vivax* infection in pregnancy. The variation in *Plasmodium* species among these two regions is mainly attributed to lack of Duffy-binding proteins that are absent among majority of African population making them resistance to *P. vivax* infection as this protein is required by the parasite to invade reticulocytes.

Diagnosis is established by microscopic examination of peripheral blood smear and by rapid antigen detection kit. However, parasitemia is difficult to establish by microscopy due to sequestration of infected malarial parasite in placenta. Thus, molecular techniques such as PCR, real-time PCR are promising tools that can be utilized in future. Major disadvantage of molecular techniques is that these are costly and require sophisticated laboratory equipment. However, loop-mediated isothermal amplification (LAMP) could provide an answer to such a problem as it can be performed in simple water bath and does not require gel-documentation system as amplified products can be visualized by naked eye.

As majority of pregnant females suffering from malaria are asymptomatic, intermittent preventive chemotherapy is advocated in high transmission areas. However, due to increase in emergence of drug resistance, safety and efficacy of artemisinin-based combination therapy and newer antimalarials should also be established in near future along with implementation of other vector control measures. Research is also required to develop new antimalarials, which are safe in pregnancy for the radical cure of *P. vivax* infection.

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Chapter 4

Challenges of Managing Childhood Malaria in a Developing Country: The Case of Nigeria

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65488

Abstract

Malaria still remains one of the highest childhood killer diseases, especially in the developing countries of Africa, Southeast Asia, and Eastern Mediterranean regions. With an estimated 100 million cases and 300,000 deaths from malaria annually, Nigeria has one of the highest burdens of malaria in the world, with children mostly affected. It accounts for 60% of outpatient visits, 30% hospitalization among children under 5 years of age. Great efforts and huge funding have been committed globally towards the fight for malaria, but malaria continues to be a major challenge in these developing countries, especially countries in Sub-Saharan Africa. The World Health Organisation adopted a cost-effective intervention strategy, which comprises a three-pronged approach: vector control, chemoprophylaxis, and case management. Case management involves early diagnosis and treatment. This chapter looks at the challenges militating against the achievement of this important aspect of malaria control in children as well as efforts that have been made or not made to overcome these challenges using Nigeria as a case study.

Keywords: malaria, childhood, challenges, managing, Nigeria

1. Introduction

Malaria still remains one of the highest childhood killer diseases, especially in the developing countries of Africa, Southeast Asia, and Eastern Mediterranean regions. In Africa, it is estimated that malaria kills one child every 2 minutes [1]. According to the latest estimates from the World Health Organisation (WHO), there were 214 million new cases of malaria worldwide in 2015 with an estimated 438 000 malaria deaths [1]. Most of these cases (88%) and deaths (90%) were recorded in the African region [1]. Nigeria and the Democratic Republic

of Congo, both in African region, accounted for about 40% of the total estimated malaria deaths worldwide. The Southeast Asia and the Eastern Mediterranean regions account for the few remaining cases in that order. The denominator in all these regions, apart from the favourable weather conditions for the breeding of malaria parasite-carrying mosquitoes, is the widespread poverty and systemic inadequacies: lack of political will and poor health systems in many of the countries that make up the regions. In parts of the world where malaria is endemic, severe malaria is mainly a disease of children under the age of 5 years due to the acquisition of specific immunity against plasmodium; the malaria-causing parasite as the child gets older, providing some protection (though incomplete) against malaria [2]. Malaria is endemic in most countries of Sub-Saharan Africa with the children and pregnant women population being the most vulnerable to the disease. Of the estimated 438,000 malaria deaths worldwide, children under the age of 5 years constitute 306,000 (70%) [1].

Despite these seemingly very high figures, there has been marked decline in the mortality from malaria. The global cases of malaria declined by 18% from an estimated 262 million in the year 2000 to 214 million in 2015. The death rate decreased by 48% from a global estimate of 839,000 in year 2000 to 438,000 in 2015. In children under the age of 5 years, the number of malaria deaths fell from an estimated 723,000 in year2000 to 306,000 in 2015, a decrease of about 58% (**Table 1**). This has come through the combined efforts of the World Health Organisation, its Rollback Malaria Partners and the various governments in the different countries afflicted by the malaria scourge.

WHO region	Estimated malaria deaths by year				Percentage reduction
	2000	2005	2010	2015	2000–2015
Africa	694,000	5910,000	410,000	292,000	−58%
Americas	400	300	300	100	−66%
Eastern Mediterranean	5300	5200	2000	2200	−58%
European	0	0	0	0	0
Southeast Asia	19,000	16,000	14,000	10,000	−49%
Western Pacific	4,700	2,000	1,600	1,500	−68%
World	723,000	614,000	428,000	306,000	−58%

Source: World Malaria Report 2015.

Table 1. Estimated number of malaria deaths in children under the age of 5 years from year 2000 to 2015 by the WHO region.

To achieve this, the WHO adopted a cost-effective intervention strategy, which comprises a three-pronged approach: vector control, chemoprophylaxis, and case management. The vector control involves the prevention of mosquitoes from acquiring or transmitting infection through the use of long lasting insecticidal nets (LLIN) and indoor residual spray (IRS). Chemoprophylaxis involves the use of antimalarial drugs to suppress and prevent the establishment of

http://dx.doi.org/10.5772/65488

infection in the human, whereas case management involves the prompt diagnosis and treatment of malaria.

Although these strategies are said to be cost effective, the achievements recorded in the fight against malaria has not come at a cheap price. The global cost of fighting malaria increased from the sum of US$ 960 million in year 2000 to a whopping US$ 2.5 billion in 2014 [1]. The majority of these funds were contributed by international partners (donor agencies). In spite of these global efforts and huge funding for malaria control, malaria continues to be a major challenge in these developing countries, especially countries in Sub-Saharan Africa. These challenges in the fight against malaria, with particular attention to case management, are discussed below using Nigeria as a case study.

2. Nigeria and malaria burden

As the most populous country in Africa, Nigeria contributes the majority of the Africa malaria burden. The country has an estimated total population of approximately 172 million (2014 estimate) with an annual growth rate of about 2.7% and a GDP per capita of US$1091.64 (2014) and inflation rate of 9.0% (2015) [3]. The public health sector in Nigeria is run by the three tiers of government: federal, state, and local governments. The federal government co-ordinates the affairs of the tertiary health facilities and provides overall policy and technical support to the health sector. The state government co-ordinates those of the secondary centres and some state-owned tertiary centres and also lend support to the primary health sector. The local government takes care of the primary health centres, which have gone moribund in many parts of the country. The annual health budget is usually well below 10% of the total budget. For instance; in 2015, it was 6.3%, whereas in 2016, it dropped to about 3.7%. In general, the country's health system may be described as weak due to inadequate funding and supervision.

Nigeria, being a tropical country, has one of the best combinations of adequate rainfall, temperature, and humidity, allowing for the breeding and survival of the female anopheline mosquitoes, the malaria vector. Most (97%) of the malaria in Nigeria are mainly due to *Plasmodium falciparum*, with *Plasmodium ovale* and *Plasmodium malariae* affecting only a few persons. Although, severe malaria is mostly caused by *P. falciparum*, *Plasmodium vivax*,and *Plasmodium knowlesi* have also been indicated in causing severe disease [2]. However, *P. vivax* does not occur in indigenous Nigerians [4], and there is no documentation of *P. knowlesi* in any Nigeria.

Malaria remains a major public health problem in Nigeria. It accounts for more cases and deaths than in any other country in the world. There are an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria [5]. Malaria accounts for 60% of outpatient visits and 30% of hospitalizations among children under 5 years of age in Nigeria [5]. This implies a huge loss in terms of financial resources and man hours and in the case of the school age child, loss of school hours [6–8].

In line with the World Health Organization recommendation, the country has adopted the Test, Treat, and Track (3T) strategy with all suspected cases of malaria properly diagnosed

using Rapid Diagnostic Tests or microscopy, treated promptly with recommended artemisinin-based combination therapy (ACT) if the result is positive and documented [4].

3. Malaria diagnosis

3.1. Clinical diagnosis

WHO has identified early diagnosis as the first step in the treatment of malaria. This is necessary to prevent progression of the disease to severe forms. Malaria does not have any specific symptoms and signs and as such may mimic several tropical childhood illnesses [9]. These include bacterial sepsis, enteric fever, pneumonia, meningitis, urinary tract infection, otitis media, and pharyngitis. This makes the specificity of clinical diagnosis of malaria very poor and thus gives room for indiscriminate use of antimalarials especially in endemic areas. In some cases, malaria may co-exist with any of these other conditions [10–13], necessitating more competent health personnel in the management. Studies [14–16] have shown that treatment based on clinical information alone leads to over diagnosis and over treatment of malaria. In addition, there may be increased morbidity and possible death from the neglected disease causing the child's symptoms.

Even with the unreliability and consequences of reliance on clinical information alone for malaria diagnosis, a large proportion of healthcare providers in Nigeria still treat malaria presumptively [14, 17, 18]. The 2013 Nigeria Demographic and Health Survey indicated that the average rate of antigen or parasite detection in children before treatment was 11.1% [19]. Reasons adduced for the presumptive diagnosis by the healthcare providers included delay in getting a blood microscopy from hospital laboratories, unavailability of malaria rapid diagnostic test (mRDT), reliance on blood tests create an impression of incompetence of the health personnel, as well as malaria being the first suspect in any child with fever since malaria is endemic in Nigeria [17]. According to WHO, less than half of the suspected malaria cases in most malaria endemic regions are truly infected with malaria parasite, hence their insistence on laboratory confirmation before treatment [1].

3.2. Parasite detection

In line with the WHO policy, the national malaria guideline of Nigeria recommends the confirmation of all suspected cases of malaria by parasitological testing either with microscopy or by the use of mRDT. This is particularly important as accurate diagnosis before treatment will enhance a more targeted therapy, reduce the duration of illness, and may even prevent death from treatment of another disease as malaria. It will help prevent drug resistance and also enhance the diagnosis and case management of other diseases that may mimic malaria in children since the exclusion of malaria will mean a search for the cause of illness. Apart from saving lives, both money and man hours are saved.

The challenge with this confirmation test is mainly that of availability and willingness to go for test before treatment. Microscopy, which still remains the gold standard, is fast disappearing

http://dx.doi.org/10.5772/65488

from many health facilities. The reasons range from inability to acquire new or replace old and damaged microscopes to absence of a qualified microscopist. In addition, ready availability of reagents and slides for use with the microscopes, where available may be a problem. There is also the need for quality assurance, which is virtually non-existence in most health facilities in the country. Another important consideration is the need for electrical power, which is a big issue in the country. The alternative is the mRDT, which is an antigen detection test. Three antigens: *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2), plasmodial aldolase, and plasmodial lactate dehydrogenase (pLDH) are currently used for mRDTs. The *pf*HRP2 mRDTs are the most commonly available types in Nigeria and seem most suited for the country as up to 97% of the malaria cases are due to *P. falciparum*. The test is easier to conduct and requires neither power supply nor any expertise. It is usually available as a pre-packed ready-to-use kit with a single container of buffer solution in every box of kits. Since the adoption of the mRDT by the WHO, the number of the products has continued to increase rapidly with over 200 different products available in the market worldwide [20]. This rapid proliferation of brands also lead to increase in the number of substandard products, which easily find their way into developing countries like Nigeria with less stringent quality control. Reviews and studies show that the commercially available MRDTs show wide variability in diagnostic sensitivity and specificity [21–25]. To stem this "influx," WHO has continued to conduct testing of the products and issuing guidelines to assist countries adopt the mRDT for use [20].

Since the introduction of RDT kits, there has been increased knowledge of mRDT by healthcare givers and increasing availability at health facilities, but its regular utilization to confirm suspected cases of malaria by these health workers prior to treatment is not encouraging [17, 26]. Another major concern is the fact that a good number of these mRDTs have high false negative results at lower antigen titre (200–500 parasites/ml) [20, 23]. For the detection of *P. falciparum* in all transmission settings, WHO recommended the panel detection score against *P. falciparum* samples should be at least 75% at 200 parasites/μL [27]. The Nigerian malaria treatment guideline recommends the use of an alternate diagnostic test where continued suspicion exists after a negative test [4]. Some authors have recommended the combined use of two methods to confirm the diagnosis of malaria [22]. This invariably adds to the cost of treatment as well as time spent at the health facility, which many healthcare givers and patients will evade. Also, quality and performance check of the mRDTs is partly absent or non-existent in malaria-endemic countries, including Nigeria [20]. The quality of the mRDT may be affected the very high temperatures (above 40°C) such as can be found in some parts of the country. It is important to note that although mRDT may constitute an extra cost to the case management of malaria, it is no doubt a cost-effective innovation, even more cost effective than microscopy [28]. Despite these challenges with the use of mRDT and microscopy, they are still very useful components of malaria case management.

4. Malaria treatment

In consonance with the WHO recommendation on the treatment of malaria, the Nigerian government adopted the use of artemether-lumefantrine (AL) for the treatment of uncompli-

cated malaria and artesunate-amodiaquine (AA) as alternative [4]. For severe falciparum malaria treatment, the drug of choice is intravenous artesunate with parenteral artemether or quinine as alternatives where intravenous artesunate is not available. For settings where a complete treatment of severe malaria is not feasible, a pre-referral treatment with intramuscular or rectal artesunate or intramuscular quinine in this order of preference is recommended.

Although the Nigerian government had adopted AL and AA as the first and second line respectively, several brands of other ACTs are widely available and used in the country. These include artesunate-mefloquine, dihydroartemisinin-piperaquine, artemisinin-piperaquine, and artesunate-sulfadoxine pyrimethamine. Regardless of the constellation of ACT antimalarial drugs available, many children still receive monotherapy notably chloroquine and sulfadoxine-pyrimethamine especially among the lower socioeconomic class and those with uneducated or under educated parents [17, 29]. Other commonly used antimalarial monotherapy in children include oral artesunate, amodiaquine, halofantrine, and oral quinine and paludrine [29, 30]. The drawback in this practice is the progression to severe malaria with its consequences, which include prolonged hospitalization, increased cost of treatment, avoidable blood transfusions, and even death. The frequent prescription of concomitant medications in the form of different antipyretics, sometimes multiple, haematinics, multivitamin preparations, and antibiotics, which are mostly inappropriate also add to the cost of treatment and burden of medications for the child [29]. In Sub-Saharan Africa, the proportion of children aged under 5 years, who received an ACT, is estimated to have increased from less than 1% in 2005 to 16% in 2014, but still falls substantially short of the target of universal access for malaria case management [1]. One important reason for this poor situation is that a greater proportion of children with fever are treated outside the health sector.

In Nigeria, only about 35% of children receive treatment at government health facilities [19]. The rest are treated by the patent medicine vendors (PMV), pharmacy shops, private health facilities, or by traditional medicine practitioners. In the light of this, the government of Nigeria with the assistance of Roll back Malaria partners and donor agencies tried to scale up the treatment of malaria through the engagement of the private sectors, especially the PMVs whom they have trained in some states of the federation and the provision of pre-packaged, age-specific antimalarials for easier dosing [19]. A study by Berendes et al. [31] in the northern part of Nigeria showed that patent medicine vendors are not reliable in the treatment of malaria because most PMVs were ignorant of and lacked training about new treatment guidelines that had endorsed ACTs as first-line treatment for uncomplicated malaria. They stocked and dispensed monotherapy to patients with suspected malaria. However, another study [32] in the southern part of the country showed an improved knowledge of ACT by PMVs, but yet other non-recommended antimalarial drugs such as chloroquine were still sold to customers without prescription. Another worrisome trend is the prevalent use of various forms of herbal medications in the treatment of malaria even in urban areas [7, 33, 34]. Some of these herbal preparations, which mostly do not have standard dosage, have the potential to cause liver or kidney damage [35].

The cost of malaria treatment is also a contributing factor to the non-use of ACT because this remains high for the average Nigerian. In 2009, the cost of treating a child was about US$6.58,

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which was mostly on out of pocket expenses [8]. Although a national health insurance scheme is available, only a very small percentage of the population, mainly federal civil servants and staff of big companies, have access to it.

Another major challenge with the treatment of malaria in Nigerian children is the high rate of fake and substandard antimalarial drugs [36, 37]. This brought about a lot of confusion to the healthcare provider regarding the possibility of co-morbidities when the child failed to respond to the proper treatment. This may lead to the administration of unwarranted antibiotics while the child deteriorates from untreated malaria. However, the situation has improved significantly in recent times [36]. The rate of fake and substandard drugs in the country improved from about 41% in 2002 to about 10% in 2010 [36]. The fight is still ongoing by the country's National Agency for Food and Drug Administration and Control (NAFDAC) to eradicate fake drugs from the country.

5. Other challenges

5.1. Co-morbidity with chronic diseases

Nigeria being a developing country has its fair share of children with chronic diseases, such as malnutrition, HIV/AIDS, and sickle cell anaemia. These children equally suffer from malaria, which is usually more severe than in the normal child [38–40]. Managing such children could pose a great challenge. Apart from the complications such as severe anaemia, which are more prevalent in these children [38–40], concurrent management of the underlying disease in the face of challenging finances often result in difficult decisions.

5.2. Blood transfusion

Children with severe malaria anaemia suffer greatly due to the difficulty in obtaining safe blood for transfusion. The primary health facilities and many private facilities do not have logistics to store blood. Patients requiring blood transfusions in these facilities are either referred to tertiary centres or made to purchase blood from private blood banks at very exorbitant prizes, some of which the safety are not guaranteed. In some cases, the child may be unable to make it alive to the referred centre. Those who do often face many logistic challenges ranging from cost of processing to provision of donor to replace the transfused blood.

5.3. Delayed presentation

Late presentation usually results in delay in initiation of the right and adequate treatment. No doubt this contributes significantly to the malaria mortality in children. Factors responsible may include poverty, inadequate home treatment, and lack of access to standard treatment centres. Certain traditional beliefs such as association of fever with teething and growth may also delay presentation. Likewise, the increasing belief in unorthodox treatment such as herbal medications and faith-based healing may also lead to delays in presentation.

5.4. Geographic inequality of health facilities and personnel

Although majority of Nigerians reside in the rural areas, only a small proportion of health facilities are located there and these are mainly primary and secondary care facilities. Likewise, fewer personnel are available in these rural communities. As a result, most patients with severe forms of malaria will need to travel some distance with all the attendant challenges such as bad roads.

6. Conclusion

Despite all these numerous challenges plaguing the management of malaria in Nigerian children, there have been significant gains in the fight against malaria as evidenced by the improved indices. Thanks to all the Rollback Malaria partners operating in the country. However, a lot of commitment and willingness to translate what is on paper to action is still expected from the Nigerian government.

First is to strengthen the health systems at all levels from primary to tertiary in order to restore the hopes of the numerous Nigerians who die because they cannot afford standard private health facility treatments. This should include the recruitment and adequate training and retraining of healthcare personnel in all the primary and secondary care centres to be able to make proper diagnosis and treat malaria cases. Also, adequate equipping of all the government health facilities with diagnostic facilities and eradication of the "out of stock syndrome" for antimalarial drugs.

There should be strengthening of the already on-going awareness creation through the media to sensitize the public on the need to seek care early and at government approved health facilities.

There should be continuous appraisal of the antimalarial drugs and mRDTs in the country with a view of identifying and expunging the substandard and fake ones. The large number of different brands of ACTs and in the country need to be pruned down to just a few quality and trusted brands.

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Chapter 5

Multiple Organ Dysfunction During Severe Malaria: The Role of the Inflammatory Response

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65348

Abstract

Severe malaria is a systemic illness characterized by the dysfunction of one or more peripheral organs, such as the lungs [acute respiratory distress syndrome (ARDS)] and kidneys [acute kidney injury (AKI)]. Several clinical and experimental studies suggest that features of the inflammatory response are related to the multi-organ dysfunction observed in severe malaria. Our group has been dedicated to studying the roles of pro- and anti-inflammatory mediators in the multi-organ dysfunction observed in experimental severe malaria, especially in the lungs, kidneys, and brain. Herein, we explore severe malaria as a pathology derived from intense inflammatory responses in different organs and further distinguish and compare these organ-specific inflammatory responses. The pathophysiological mechanism of severe malaria is not fully elucidated; however, it is important to study it as a complex inflammatory response assembled by different actors, each one orchestrating a different mechanism.

Keywords: inflammation, cerebral malaria, acute respiratory distress syndrome, acute kidney injury, vascular permeability

1. Introduction

Severe malaria is a systemic illness characterized by one or more clinical manifestations, such as acute respiratory distress syndrome (ARDS), multiple convulsions, prostration, shock, abnormal bleeding, jaundice, and acute kidney injury (AKI) [1–3]. Severe malaria used to be exclusively attributed to *Plasmodium falciparum* infection. However, in the last 15–20 years,

several reports of severe malaria attributed to *Plasmodium vivax* [4–6] and *Plasmodium knowlesi* [7–9] have been described, which led the World Health Organization (WHO) to add these species as causes of severe malaria [10]. According to the WHO, severe malaria evolves from an uncomplicated illness due to several factors, such as the host response, parasite virulence, comorbidities, and deficient health services for malaria patients. Beyond the three species cited above, *Plasmodium malariae* and *Plasmodium ovale* also affect multiple organs in children and adults, however with different intensity (**Table 1**). The multi-organ dysfunction observed during severe malaria is associated with a systemic inflammatory response triggered by, among other factors, leukocyte adhesion to organ microvasculature, parasitized erythrocytes and production of inflammatory mediators [11, 12]. Despite the morphological and biochemical differences among *Plasmodium* species, the mechanisms by which severe malaria develops appear to be similar. Herein, we discuss the inflammatory response underlying the Physiopathology of severe malaria in human and experimental data. We further discuss triggers of the inflammatory response and how chemical and cellular mediators of inflammation cause severe malaria-induced multi-organ damage [6, 7, 9, 13–36].

	Clinical manifestation			
	ARDS	CM	Jaundice	AKI
Species				
P. falciparum	[13, 14]	[13, 15–22]	[13, 14, 16, 17, 23]	[13, 14, 16, 18, 24]
P. vivax	[6, 23, 25–27]	[27]	[6, 23]	[6, 18, 27]
P. knowlesi	[9, 28, 29]	–	[32, 36]	[31, 32, 36]
P. malariae	[31]	[6, 18, 27]	[7, 28–30]	[31, 32, 36]
P. ovale	[31, 33, 34]	–	[33, 35]	[31, 35]

ARDS, acute respiratory distress syndrome; CM, cerebral malaria; AKI, acute kidney injury.

Table 1. Studies describing severe malaria clinical manifestation according *Plasmodium* species.

2. Molecular and cellular features of the malaria-induced inflammatory response

During severe malaria, leukocytes and lymphocytes produce soluble inflammatory mediators, such as pro-inflammatory cytokines, which activate endothelial cells [37]. Furthermore, proteins anchored on membranes of infected red blood cell (RBC) such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed by parasites, induce endothelium activation resulting in increased expression of adhesion molecules [38, 39] and the activation and adhesion of leukocytes to the microvasculature.

In both the pre-erythrocytic and erythrocytic phases, macrophages and monocytes are responsible for the cytokine storm during an acute malarial infection [40]. Activation of

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phagocytes is mediated by binding of the hemozoin/parasite DNA complex to TLR-9 and the consequent downstream activation of inflammasome signaling [41]. The hemozoin released into circulation during infected RBC lysis is taken up by circulating monocytes and tissue macrophages and activates inflammasome intracellular protein complexes, such as NOD-, LRR-, and pyrin domain-containing (NLRP)3 and NLRP12, resulting in caspase 1 activation and the subsequent release of interleukin (IL)-1β, which is involved in fever during malaria bursts [40, 42]. In addition to inducing pro-inflammatory cytokines, some studies demonstrate that hemozoin can also induce the expression of anti-inflammatory cytokines in monocytes, such as IL-10, which tightly regulates IL-12 and CCL5 production [43]. These cytokines and chemokines, respectively, are directly involved in the development of the immune response [44]. Mononuclear cell activation leads to the production of TNF-α and IL-12 by neutrophils. These cytokines stimulate innate immune cells, such as natural killer (NK) cells and γδ T cells (including γδ NKT cells), to rapidly produce IFN-γ. As a consequence, IL-12 and IFN-γ activate monocytes and macrophages to enhance the phagocytosis of infected RBCs (reviewed in [45, 46]) and produce reactive oxygen and nitrogen radicals, which kill parasites [47].

The activation of the cellular components of the innate immune system, such as dendritic cells (DCs), is important for the establishment of acquired immunity [40]. In the spleen, DCs present their processed antigens to naïve T cells (Th0) and induce a pro-inflammatory response (Th1) with mainly $CD4^+$ T cells that produce IFN-γ. This lymphocyte subtype is involved in the beginning of malarial infection by further stimulating Th1 differentiation and subsequently stimulating B cells to produce specific antibodies to eliminate malaria parasites [46]. In addition, $CD8^+$ T cells act in the effector phase, contributing to permeability changes in the blood-brain barrier (BBB) through perforin-dependent mechanisms [48].

Beyond leukocytes and lymphocytes, endothelial cells also play a crucial role in the inflammatory response during severe malaria. In the erythrocytic phase, endothelial activation accounts for many factors involved in the development of severe malaria [49], such as increased adhesion of infected RBCs [50], increased expression of chemokines [51], and increased adhesion of leukocytes to peripheral organ microvasculature [52]. Several soluble proteins have been described such as inflammatory markers of endothelial activation during severe malaria. The angiopoietin (Ang)-Tie2 axis is a critical regulator of endothelial quiescence, activation and dysfunction in infectious and oncologic diseases, atherosclerosis, and pulmonary hypertension [53, 54]. Ang-1 signals through its cognate receptor Tie-2 (a tyrosine kinase with immunoglobulin and endothelial growth factor homology domains), which is expressed on endothelial cells [53]. In addition, Ang-2 (partial/weak agonist of Tie-2) is released by endothelial cells and acts as an Ang-1 antagonist [55]. During cerebral malaria (CM), Ang-1 exerts anti-inflammatory effects by decreasing adhesion molecule expression and maintaining the integrity of the BBB by reinforcing VE-cadherin tight junctions [53, 54]. In contrast, Ang-2 is stored in Weibel-Palade bodies (WPB) within endothelial cells and is involved in the response to inflammatory stimuli. High levels of Ang-2 are observed in children with severe malaria [56]. In healthy subjects, the basal Ang-1 level is higher than that of Ang-2, while the opposite ratio is observed in fatal cases of severe malaria [57]. Another inflammatory marker of endothelial activation during sever malaria is the activation of endothelial cell protein C

receptor (EPCR). EPCR is widely expressed on endothelial cells and leukocytes, and its activation is associated with severe malaria [58, 59]. EPCR is referred to as the cell surface conductor of cytoprotective coagulation factor signaling because it enhances the conversion of protein C into its activated state, activated protein C (APC). The EPCR/APC complex has anti-inflammatory and endothelial cytoprotective activities that help maintain vascular integrity [60, 61]. The binding of infected RBCs to EPCR impairs the formation of the EPCR/APC complex, which may lead to sequestration, complement activation, and endothelial dysfunction, as reflected by Weibel-Palade (WP) body exocytosis, with the release of von Willebrand factor (vWF) and angiopoietin-2 and the increased expression of other endothelial receptors, such as ICAM-1 [60].

3. Organ-specific inflammatory responses

The inflammatory features described above occur in different organs and at different intensities. Although there are few examples of leukocyte adhesion in the brain vasculature in the development of human cerebral malaria [62], necropsy in fatal cases of severe malaria reveals marked inflammatory cell infiltration in lung tissue [11]. Endothelium/leukocyte interactions in the lung differ from their interactions in the brain, likely due to differences in the BBB and the blood-air barrier tight junction compositions of the brain and lung endothelium. However, the malaria-induced inflammatory response that is responsible for kidney dysfunction is not related to inflammatory cell accumulation in renal tissue but depends on immunocomplex deposition and infected RBC adhesion to the renal vasculature [63].

3.1. Inflammatory components in the development of cerebral malaria

Cerebral malaria is mainly attributed to *P. falciparum* infection, especially in children under five years [64]. Cerebral complications during malaria are triggered by the mechanisms described above; however, the inflammatory response observed in the brain is unique.

Taylor and coworkers have been studying the pathogenesis of cerebral malaria (CM) and have observed three different pathologies: (i) CM1—presence of sequestered parasitized erythrocytes in the cerebral microvasculature; (ii) CM2—presence of sequestered parasitized erythrocytes in the cerebral microvasculature and vascular pathology; and (iii) CM3—non-malarial components involved in cerebral damage. Inflammatory mediators are involved in CM1 and CM2. As described above, adhesion molecules and EPCR expressed in brain endothelial cells induce parasitized erythrocyte adhesion [58]. Likewise, during CM2, leukocytes are observed in the intravascular space, and plasmatic proteins are found in the brain tissue, suggesting edema formation [62]. The role of leukocytes in the pathogenesis of cerebral malaria is unclear. A main characteristic of brain anatomy is the presence of the BBB, which confers protection against circulating cell diapedesis into brain tissue. Nevertheless, the BBB composition of postcapillary venules allows leukocyte diapedesis during non-malarial brain injury [65, 66]. However, leukocytes are not observed within brain tissue during CM2 [62, 67], suggesting an indirect contribution of these cells to the development of cerebral malaria. Cytokine production

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by leukocytes during *P. falciparum* infection may contribute to brain endothelial cell activation, indicating that leukocyte involvement in cerebral malaria does not depend on cell-cell contact [68, 69]. Wassmer and colleagues hypothesized that higher endothelial responses to TNF-α increase the probability of a patient developing cerebral malaria. The authors suggest that endothelial activation by TNF-α increases the expression of adhesion molecules, which facilitates the binding of parasitized erythrocytes, leading to CM1/CM2. Thus, CM1/CM2 is a pathogenesis triggered by parasitized erythrocytes but sustained by a local inflammatory response (**Figure 1**).

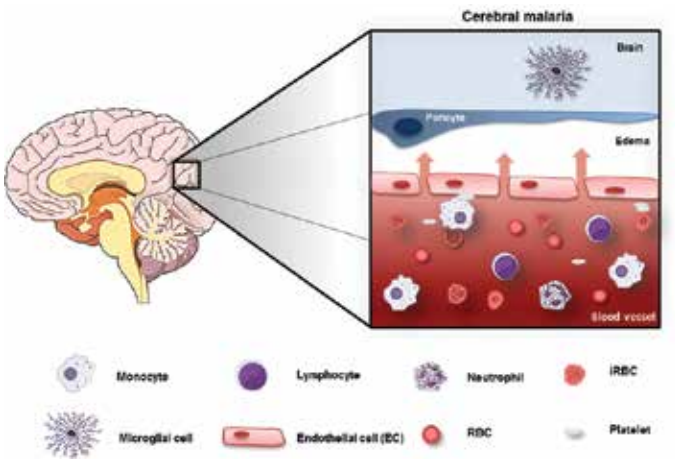

Figure 1. Inflammatory response during cerebral malaria—during cerebral malaria, it is possible to observe the presence of sequestered parasitized erythrocytes in the cerebral microvasculature, vascular pathology, leukocytes in the intravascular space and plasmatic proteins in brain tissue, suggesting edema formation. Figure created in the Mind the Graph platform (www.mindthegraph.com).

Although experimental models of severe malaria could not be used to predict human pathology, they have been extensively used to elucidate cellular and molecular pathophysiological processes. Several findings observed in human cerebral malaria are also observed in experimental models, including cytokine activity [70], endothelial activation [71], and edema formation [72]; however, the sequestration of parasitized erythrocytes during experimental cerebral malaria (ECM) is not well understood. Recent evidence showed that *Plasmodium berghei*-ANKA infected RBCs adhere to brain microvascular endothelial cells in a VCAM-1-dependent manner [73]. In addition, another study suggests transient contact between infected RBCs and the endothelium [74]. The expression of Pf-erythrocyte membrane protein (EMP)s and their ability to adhere to host adhesion molecules depends on the expression of structural proteins, such as knob-associated histidine-rich protein (KAHRP), that allow the formation of knobs on erythrocyte membranes [75]. *Plasmodium* species incapable of forming knobs in infected erythrocytes (knobless *Plasmodium*) show a passive adhesion of infected RBCs to activated endothelial cells [75]. Thus, knobless *Plasmodium* activates endothelial cells to the same extent as knob-forming *Plasmodium* [66, 73], which suggests that ECM may also be induced by parasitized erythrocytes.

The participation of leukocytes and lymphocytes in ECM has been extensively described [76]. Different from that observed in humans, during ECM, the adhesion of leukocytes and lymphocytes in the brain vasculature is well described [71, 74, 77]. In fact, monocytes, $CD4^+$ T cells, $CD8^+$ T cells and platelets adhere in brain post capillary venules but do not transmigrate to the brain tissue of *P. berghei* infected mice, supporting the idea that the brain disorder is due to leukocyte induced-endothelial dysfunction. Thus, strategies targeting endothelial stabilization revert ECM and prolong survival in mice [71, 78].

3.2. The inflammatory response in severe malaria-induced ARDS

Beyond the brain, the lungs are the most affected organ in severe malaria. Lung dysfunction occurs in 20% of all cases of adults with falciparum [3] or vivax [27] severe malaria. In knowlesi severe malaria, more than 50% of patients develop acute respiratory distress syndrome (ARDS) (reviewed in [3]). Recently, the methods for ARDS diagnosis are redefined, and ARDS is now classified as mild, moderate, or severe according to chest imaging, the origin of edema, oxygenation, and respiratory dysfunction timing [79], which supports the idea that the epidemiological data regarding malaria-induced ARDS may be underestimated. Nevertheless, ARDS can be caused by direct lung injury (pulmonary infection, aspiration, lung contusion, etc.) or by indirect lung injury (systemic inflammation, transfusion, burn injury, etc.) (reviewed in [80]). Thus, during severe malaria, lung dysfunction can be triggered directly by adhesion of infected RBCs to the lung vasculature or indirectly as a consequence of the activity of endothelial activators (**Figure 2**).

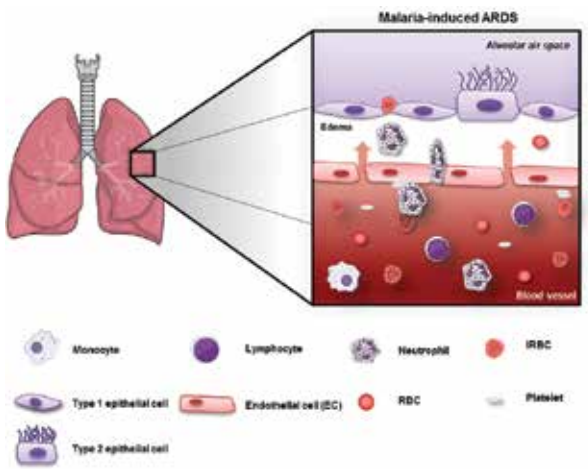

Figure 2. Inflammatory components observed in severe malaria-induced ARDS—in the lungs of patients with severe malaria who develop ARDS, increases in vascular permeability, infected erythrocytes, and intense neutrophil infiltration are often observed. Figure created in the Mind the Graph platform (www.mindthegraph.com).

Although CM is common in children, ARDS is often observed in adults [81]. In fact, the pathology observed in the lung tissue differs between adults and children. In children, few cases of pneumonia are observed [11], while an intense inflammatory cell infiltration is

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frequently noted [11, 82]. Milner and coworkers hypothesize that ARDS in children is an indirect effect of the inflammatory response induced by CM because non-specific lung dysfunction is observed. In fact, it has already been demonstrated that the inflammatory response triggered by brain injury directly affects the respiratory system by altering vascular permeability and allowing leukocyte influx into the lung parenchyma [83]. However, in adults, the presence of infected RBCs likely induces a local inflammatory response. Gillrie and coworkers proposed that merozoite-derived histones bind to pathogens-associated molecular patterns (PAMPs) expressed on endothelial cell membranes, leading to MAPK activation and the consequent production of pro-inflammatory mediators. In addition to the production of inflammatory mediators, *Plasmodium* also induces cell death and alterations in the expression of junctional proteins, which facilitates the influx of leukocytes to pulmonary tissue [84, 85].

Experimental models of severe malaria have revealed that ARDS begins when merosomes activate endothelial cells within pulmonary capillary beds [86, 87]. Thus, some authors suggest that the erythrocytic cycle starts in the lung capillaries [86]. In addition to merosomes, hemozoin and the close contact between infected erythrocytes and pulmonary endothelial cells trigger an inflammatory response 24 h after infection. This is characterized by intense leukocyte infiltration, as well as the production of proinflammatory mediators in the lung tissue, which persists for at least five days after infection [88–91]. Different from that observed in brain pathology, the inflammatory cellular infiltration in the lungs is mainly composed of neutrophils [90]. In fact, depletion of neutrophils impairs experimental severe malaria-induced ARDS and prolongs survival in mice [92, 93]. The participation of leukocytes in lung dysfunction during malaria may be explained, in part, by their interaction with the endothelium. In the brain, there is no leukocyte transmigration, while in the lung, tight junctional constitution and adhesion molecules expressed in the endothelium allow leukocyte transmigration and the consequent accumulation of these cells in the lung parenchyma. Thus, despite constitutional differences, the preservation of endothelial integrity in both the lungs and the brain may contribute to the attenuation of severe malaria symptoms.

3.3. The inflammatory response observed in severe malaria-induced acute kidney injury

Systemic disorders often result in secondary damage, such as functional and structural changes in the kidneys and consequent acute renal failure (ARF). The term ARF was replaced by the term acute kidney injury (AKI), which represents more than renal failure characteristics, according to the risk, injury, failure, loss, and end-stage renal failure (RIFLE) criteria [94, 95]. At present, the RIFLE criteria are widely used to diagnose AKI [96]. Severe malaria-derived AKI (smAKI) is more common in adults than in children [81]. Beyond the AKI reported in severe cases of *P. falciparum* and *P. vivax* malaria [97, 98], there have previously been reports of AKI in conjunction with the rare complications derived from infection with *P. ovale*, *P. malariae*, or *P. knowlesi* [35, 99, 100]. AKI is diagnosed in almost 50% of severe malaria cases. Currently, smAKI is diagnosed according to the WHO 2006 criteria; however, Thanachartwet and colleagues suggest that, according the RIFLE criteria, these numbers are underestimated. Instead, according the RIFLE criteria, almost 75% of severe malaria patients are developing AKI [96].

The pathophysiology of smAKI is still unclear. Because AKI can develop as a secondary effect of a systemic disease, some authors suggest that the systemic inflammatory response induced in peripheral organs during severe malaria contributes to smAKI development [101]. However, ultra-structural and histological studies of renal tissue in fatal cases of severe malaria reveal an intense inflammatory cell accumulation, indicating that smAKI can also be locally induced [18, 102].

In general, endothelial cell swelling, hypertrophy, and cytoplasmatic vacuolation suggest endothelial activation and are characteristic of smAKI [18, 102]. Such characteristics are similar between affected organs [3, 62]; however, unlike brain endothelial cells [103], kidney endothelial cells do not phagocytose infected RBCs. Regarding leukocytes, smAKI is characterized by the intense presence of mononuclear cells in peritubular capillaries, but not neutrophils, platelets, or eosinophils (**Figure 3**). Increased levels of plasmatic TNF-α [104], soluble urokinase-type plasminogen activator receptor (suPAR) expression [105], and mononuclear activation markers correlate with AKI in patients with severe malaria, suggesting that mononuclear activation induces tissue damage. Furthermore, mononuclear cells do not infiltrate the renal tissue interstitium as they do in the lungs [3], likely because, despite the activation of the renal endothelium, the tight junctions in renal tissue are not fully disrupted during severe malaria [106]. Another inflammatory characteristic that is mainly attributed to AKI is the deposition of immune complexes in the kidneys. The nephropathy associated with the deposition of immunoglobulin (Ig) isotypes G and M in the kidneys has previously been described in patients with severe malaria; however, the pathological events that result in immune complex deposition depend on the *Plasmodium* species and the time of patient death [107, 108].

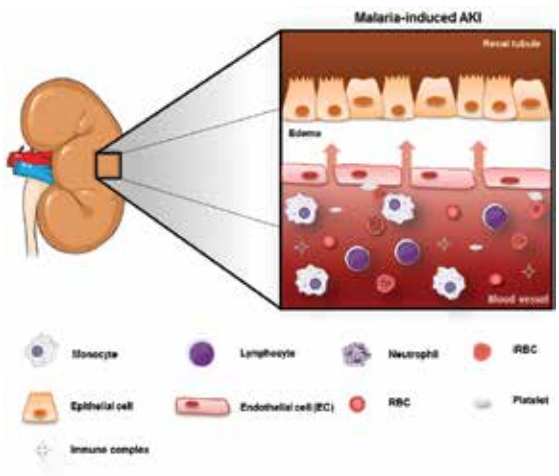

Figure 3. Severe malaria-induced AKI—during severe malaria-induced AKI, there is an intense mononuclear cell accumulation in renal tissue, endothelial cell swelling, hypertrophy, and cytoplasmatic vacuolation, suggesting endothelial activation. Different from that observed in the lungs and brain, this suggests that AKI results from deposition of immunoglobulins in the kidneys. Figure created in the Mind the Graph platform (www.mindthegraph.com).

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Inflammatory components of AKI are also observed in experimental models of severe malaria. Endothelial dysfunction assessed through the evaluation of increased vascular permeability [109] and the expression of adhesion molecules [110] is also observed in experimental models of severe malaria. The activation of the glomerular endothelium may be involved in the accumulation of inflammatory cells and infected erythrocytes in glomeruli [111]. Furthermore, inflammatory cells present in the kidneys produce pro-inflammatory cytokines that perpetuate renal damage [111]. In fact, studies in which mice were rescued from severe malaria, i.e., were cured of *P. berghei* infection, showed that renal dysfunction persists for at least 14 days after cure, suggesting that severe malaria-induced AKI is mainly sustained by inflammatory components [112].

Overall, further studies are required to unveil the pathophysiology of smAKI. To date, it is not clear how kidney tissue damage begins. SmAKI may be a secondary effect of the systemic inflammatory response, may begin locally, or may be the sum of both of these processes; however, once established, smAKI persists even after parasite clearance by antimalarial drugs [24], which raise the possibility for new therapeutic approaches that target the inflammatory response in the kidney.

4. Conclusions

The findings presented above show the influence of the inflammatory response in the development and perpetuation of severe malaria. It has been shown that *Plasmodium*-associated molecular patterns such as homozoin/parasite DNA and proteins expressed on membrane of infected red blood cells trigger inflammatory response including macrophage activation, T cell differentiation, endothelial cell activation, and the production of several pro-inflammatory mediators. *Plasmodium*-induced inflammatory response occurs systemically, however, due to different anatomical and physiological characteristics, each organ develops a particular

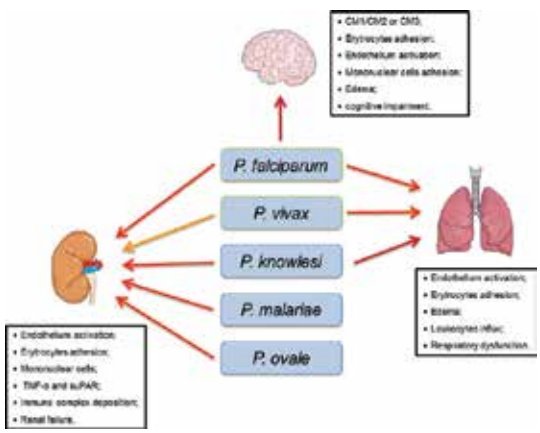

Figure 4. According to the WHO, severe malaria can be caused by *P. falciparum*, *P. vivax*, and *P. knowlesi*. However, the five *Plasmodium* species that infect humans are able to induce organ dysfunction due to a particular inflammatory response. Figure created in the Mind the Graph platform (www.mindthegraph.com).

inflammatory response that may lead to organ dysfunction (**Figure 4**). Although brain dysfunction is associated with activation of endothelial cells by the cytoadhesion of infected erythrocytes, severe malaria-induced ARDS is correlated with inflammatory cell accumulation in lung parenchyma.

Even though artemisinin derivatives are the treatment of choice for severe malaria, it accounts only for antimalarial purpose. In the last few years, host-directed therapies for malaria and other infectious diseases have been studied [113]. Several approaches aiming the inflammatory response have been studied in patients diagnosed with uncomplicated malaria [114, 115]; however, the treatment of severe malaria includes only supportive treatment. On the other hand, the use of experimental models of severe malaria suggested that the induction of cytoprotective pathways in brain as well the administration of anti-inflammatory drugs improve the survival of *P. berghei*-infected mice, especially when administrated as adjunctive treatment to antimalarial drugs [71, 76, 116, 117]. Indeed, a robust clinical evidence is yet necessary to provide the effectiveness of the treatment with inflammatory modulators as an adjunctive therapy to antimalarial drugs to improve patient outcomes.

Acknowledgements

This work was supported by grants from the Brazilian Council for Scientific and Technological Development (CNPq), Carlos Chagas Filho, the Rio de Janeiro State Research Supporting Foundation (FAPERJ), the Coordination for the Improvement of Higher Education Personnel (CAPES), and Fundação Oswaldo Cruz (FIOCRUZ).

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Chapter 6

Severe and Complicated Malaria due to *Plasmodium vivax*

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64974

Abstract

Contrary to the widespread belief that severe malaria is mainly caused by *Plasmodium falciparum*, malaria caused by *Plasmodium vivax* infection may also lead to severe clinical manifestations including a plethora of renal, pulmonary, hematologic, neurologic, and multiorgan dysfunction. Anemia and thrombocytopenia are considered as two major important markers of severity during the clinical course of severe *P. vivax* malaria. In highly endemic areas of *P. vivax* transmission, early diagnosis is crucial in preventing uncomplicated episodes progressing into severe and complicated clinical forms. In fact, given the wide geographic distribution of *P. vivax*, there is a large burden of disease, often not adequately acknowledged, and resulting from the combined effect of the large numbers of uncomplicated clinical episodes and the increasingly recognized severe and complicated clinical presentations.

Keywords: *Plasmodium vivax*, clinical manifestations, diagnosis, treatment, severe, complicated

1. Introduction

Globally, malaria remains one of the most important infectious diseases affecting humankind in terms of morbidity and mortality [1, 2]. *Plasmodium vivax* infection and *Plasmodium falciparum* represent the two most frequent and severe forms of human malaria. Individuals acquire the infection by the bite of the female *Anopheles* mosquitoes inoculating microscopic sporozoites that subsequently reach the liver via the bloodstream. Once in the liver, the sporozoites evolve into a schizont that produces merozoites and then releases them into the

bloodstream coinciding with the onset of the clinical syndrome of malaria [3]. Most importantly, most deaths associated with malaria occur in children living in highly endemic settings [3]. The large social and economic burden attributable to *P. vivax* in highly endemic settings results from the elevated number of cases that require medical care in an already impoverished economy diverting cash and family members from work to attend the sick [2, 3]. Uncomplicated *P. vivax* malaria refers to a febrile illness where there are no hemodynamic instability, no evidence of major hemolysis, no absence of severe anemia, no evidence of pulmonary edema or ARDS, and no evidence of severe metabolic acidosis, renal failure, hepatic dysfunction, focal neurological deficits or seizures, or multi-organ failure [4, 5]. Historically, severe malaria is often considered exclusively within the clinical spectrum of disease caused by infection due to *P. falciparum*. However, albeit less recognized, infection due to *P. vivax* can also lead to clinically severe manifestations and complications including (a) fatal bleeding due to traumatic or spontaneous rupture of an enlarged spleen, (b) seizures, (c) shock, (d) hepatitis with cholestasis, (e) renal failure, (f) severe anemia and thrombocytopenia, (g) respiratory distress and acute lung injury (ALI), (h) miscarriage and preterm delivery, and (h) multi-organ failure [6]. Of all these complications, the most significant source of morbidity is by far the occurrence of severe anemia and its consequences. Furthermore, in many geographic settings, coinfection of *P. vivax* and *P. falciparum* may occur in the same host, and this can be associated with severe clinical manifestations. Therefore, identifying coinfection in settings where there is co-circulation of both *Plasmodium* spp., the peripheral blood smear is of utmost clinical importance in the early course of a febrile illness in which malaria is a consideration [7]. *P. vivax* malaria was originally described clinically as "benign tertian malaria"; however, this is a misleading concept because *P. vivax* is not a benign disease. In fact, it is increasingly recognized as a major cause of morbidity and mortality in highly endemic settings.

The clinical spectrum of disease associated with *P. vivax* infection ranges from asymptomatic parasitemia and uncomplicated febrile illness to severe and fatal malaria. In this regard, the host response influences the clinical expression of the disease. Fever may occur with low parasite densities and may be identified 2–3 days before the parasites are detected in blood, underscoring the need for serial peripheral blood smear examinations [8]; *P. vivax* has a lower pyrogenic threshold (parasitic density required to evoke a fever) compared with *P. falciparum*. Globally, it is estimated that there are more than 80 million episodes of *P. vivax* malaria every year resulting in a considerable amount of morbidity and mortality. The prevailing neglect of the substantial public health impact of *P. vivax* that occurs in many settings in comparison with that of *P. falciparum* relies on its low incidence in Sub-Saharan Africa as a result of the evolutionary trait of the Duffy antigen negativity selected among many African populations [9].

P. vivax causes almost half of all of the 70–390 million clinical cases of malaria each year. In countries endemic for both major *Plasmodium* species, *P. vivax* infection may account for up to 38% of patients hospitalized with malaria. In Indonesian Papua, *P. vivax* accounted for 24% of malaria admissions in all age groups, of whom 47% (415/887) are infants [6]. The need for hospitalization among these cases indicates significant morbidity, and at least moderately

http://dx.doi.org/10.5772/64974

severe disease. Thus, clinicians should be aware of the spectrum of severe disease and of potential multi-organ affection among patients presenting with *P. vivax* malaria [10–37].

2. Parasitology and epidemiology

Infection due to *P. vivax* challenges human health in many settings, particularly in Southeast Asia, in some areas of the Pacific Islands, and in many areas in Latin America, particularly in Venezuela and Brazil [38, 39]. This type of malaria infection affects approximately 100–400 million people each year within a population living at risk of 2.5 billion. Interestingly, *P. vivax* occurs with an extremely low prevalence throughout much of Africa likely as selective pressure led to the emergence of the Duffy antigen negativity or red blood cells across African populations, particularly in West Africa. However, *P. vivax* produces substantial morbidity in some settings at subtropical and temperate latitudes in Asia [11].

P. vivax is responsible for approximately 3.3–30.3% of complications of malaria. In Colombia, *P. vivax* is responsible for almost 75% of cases of malaria. In the country, mortality from 130 to 150 cases per year is recorded, because this situation has been considered the disease as a public health concern with a growing number of complications [10, 11]. *P. vivax* accounted for 24% of malaria admissions in all age groups, including 47% of them among infants. In a recent autopsy-based report from Brazil, at least four of 17 (23.5%) *P. vivax*-associated deaths in Brazil were attributable to alternative causes at the time of death, including meningitis and yellow fever, underscoring the ability of this infection to manifest with severe disease often mimicking other conditions traditionally considered to produce severe clinical manifestations. Mortality rates in those hospitalized in Indonesian Papua with microscopy-confirmed *P. vivax* were reported as 0.8–1.6%, similar to that of *P. falciparum* infection (1.6–2.2%)). The adjusted odds ratio of death from severe anemia in Papua, Indonesia, was 5.9 for those with *P. falciparum* and 4.4 for those with *P. vivax* infection [12, 13]. Differences in intensity of transmission might contribute to the variation in the spectrum of disease severity, as it occurs with falciparum malaria. Prospective studies from Papua New Guinea conducted to address mortality associated with *P. vivax* infection have shown a mortality rate of 1.6% among hospitalized patients with *P. vivax* and of 2.2% among those infected with *P. falciparum*. These facts illustrate the similarities in terms of clinical outcomes between both types of human malaria. Further studies are needed to define *P. vivax* attributable mortality in endemic areas along with clinical studies to elucidate the precise pathophysiological and clinical spectrum of disease and from a public health perspective, the overall magnitude of morbidity and mortality associated with *P. vivax* malaria [14].

3. Pathogenesis and disease transmission

The clinical spectrum of disease of *P. vivax* infection in humans is influenced by many factors: the parasite, parasite-host interactions, host factors, and socioeconomic environment in which

these infections occur [3–5]. For example, low socioeconomic status is associated with higher risk of developing severe anemia [3]. Studies of specific organs have also shown that the inflammatory response during infection with *P. vivax* is of greater magnitude compared with that seen in *P. falciparum* infection and also with a parasite biomass similar or greater. Moreover, cytokine production in *P. vivax* infections is quantitatively more robust than in *P. falciparum* infections with a similar parasite biomass.

P. vivax infections relapse at intervals of 3–4 weeks, and progressive anemia is associated with recurrent episodes of hemolysis and dyserythropoiesis. In areas with chloroquine resistance, this is further aggravated by the elimination of delayed parasite infection and risk of reactivation. The low number of *P. vivax* parasites indicates that severe anemia may not be produced by the destruction of infected erythrocytes. This is further evident from the fact that it has been shown that during *P. vivax* malaria therapy studies have shown that for each infected erythrocyte destroyed, 32 uninfected erythrocytes are cleared from the bloodstream. This contrasts with the loss of only a few infected red blood cells during *P. falciparum* malaria. The mechanisms underlying this difference are not known; the proportion of uninfected erythrocytes destroyed compared to the proportion of extravascular subject (e.g., splenic) pooling is also not known. Recent studies show an increased fragility microfluidics of infected erythrocytes [14–16]. Pathogenic mechanisms leading to severe symptoms not known exactly, but it have suggested that a failure of the immune system to control parasite replication or immunopathological response resulting from excessive inflammation; they are considered as contributing factors in this response malaria parasites and parasite-infected red blood cells that activate dendritic cells activate dendritic. Parasite antigen presenting T helper (Th) 1 cells triggers a pro-inflammatory response. The inflammatory response required to eliminate parasites may produce tissue damage and activation of phagocytes with production of a cytokine cascade [17].

4. Clinical manifestations

When evaluating patients presenting with a clinical syndrome compatible with malaria, there are no specific signs or symptoms to assist clinicians in distinguishing infection due to Plasmodium vivax or plasmodium falciparum; or when there is coinfection by the two species. Fever in young children may produce seizures. Some individuals progress rapidly to respiratory failure caused by either pulmonary edema or even acute respiratory distress syndrome (ARDS). Among those with rapid clinical deterioration, we should always consider the concomitant risk of gram-negative bacteremias among individuals with either *P. vivax* or *P. falciparum* infection [6, 7]. The main serious manifestation in most series of vivax malaria in children is severe anemia, defined as hemoglobin < 5 g/dl in children and < 7 g/dl in adults [18]. The pathogenesis of severe malaria is caused by erythrocyte destruction after the invasion of the parasite by its multiplication and also by morphological deformity of the infected red blood cell with opsonization and antibody-dependent lysis of erythrocytes. Indeed, *P. vivax*-infected red blood cells are minimally tacky and are more deformable than *P. falciparum* erythrocytes resulting in relatively low red cell sequestration in microvasculature and bone sinuses and the

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passage of a greater proportion of erythrocytes through the reticuloendothelial spleen and other organs infected [19]. Recurrent episodes of *P. vivax* infection in highly endemic settings contribute to a higher risk of severe anemia [20, 21]. Acute respiratory distress syndrome (ARDS) occurs often in patients with severe *P. vivax* [22, 23]. Among patients with Plasmodium vivax infection and presenting with severe clinical manifestating it is important to identify splenic rupture early. In fact, the incidence of splenic rupture is as high as 24%. Factors leading to Splenic rupyure include: rapid hyperplasia and parenchymal splenic capsule, forming small infarcts bleeding, loss connective tissue fibrosis and alterations of primary immunity [6, 10].

Acute kidney injury may occur in both children and adults, and it may result for oliguric renal failure or even from acute tubular necrosis [24, 25]. Many patients present with multi-organ failure and hemodynamic instability requiring vasopressor support and often mechanical ventilation. Others may develop acute kidney injury from hypovolemic shock secondary to splenic rupture. All of these potential clinical scenarios should be considered among those with severe *P. vivax* infection and particularly among those with rapid clinical deterioration often requiring intensive care support. In some settings, *P. vivax* may present with concomitant bacterial infection including non-typhoidal salmonellosis or even with enteric fever [26–29]. Finally, patients may also develop seizure disorder or focal neurologic deficits during the clinical course of *P. vivax* mono-infection or when coinfected with *P. falciparum* [30–32]. Early recognition and institution of appropriate interventions are crucial to improve overall clinical outcomes.

5. Diagnosis of severe vivax malaria

Appropriate management of individuals with *P. vivax* requires confirmation of the diagnosis by peripheral smear examination. Coinfection with *P. falciparum* should be entertained in settings where there are co-circulations of both *Plasmodium* species [30]. Early institution of anti-parasitic therapy is instrumental. Additionally, ruling out coinfection with bacterial diseases such as non-typhoidal salmonellosis or by other enteric-gram-negative bacteria is of utmost clinical relevance. Supportive management in intensive care units should be early instituted among those with rapid progression to multi-organ failure and among those with persistent hypotension despite volume resuscitation or requiring vasopressors due to persistent hemodynamic instability. Search for potential splenic rupture needs to be considered at the time of initial presentation, or during the course of illness, among those with rapid clinical deterioration or those developing severe anemia.

6. Treatment of severe vivax malaria

A summary of core concepts in the management of severe malaria due to *P. vivax* is depicted in **Table 1**. Clinical trials to assess optimal treatment regimens of *P. vivax* malaria are limited. At this point in time, in many settings, treatment of vivax malaria consists of chloroquine or

an artemisinin-based combination therapy. In those settings where chloroquine resistance remains low, chloroquine may be used as monotherapy with continuous clinical monitoring. However, in most settings, an artemisinin-based combination therapy has become the standard of care due to the growing recognition of vivax malaria causing severe disease, also due to the risk of coinfection with *P. falciparum* (in settings with co-circulation of both species), and finally due to the increasing rates of resistance to chloroquine. Some areas consider a universal policy of treatment with an artemisinin-based combination therapy (artemether-lumefantrine) that is considered the treatment of choice since this drug combination is well tolerated and safe to pregnant women and children [34]. Additionally, there is an increasing recognition of treatment failure when using chloroquine or the combination of chloroquine/sulfadoxine-pyrimethamine [35–37].

Clinical category	Core concepts
Respiratory failure	Protect airway Provide supplemental oxygen Rule out other causes of concomitant respiratory failure (i.e., bacterial pneumonia, pulmonary embolism, or others)
Acute pulmonary edema	Oxygen supplementation BIPAP or mechanical ventilation via endotracheal intubation if needed. Patient often requires PEEP given the possibility of ARDS
Shock	Potentially caused by either hypovolemia due to bleeding or septic shock from gram-negative bacteremia Obtain blood cultures; administer broad spectrum antibiotics; correct hemodynamic disturbances Rule out splenic rupture with abdominal ultrasound or computed tomography Surgical consultation
Acute renal failure	Exclude prerenal causes Fluid replacement Hemodialysis or hemofiltration if indicated (i.e., worsening acute kidney injury, acute tubular necrosis, concomitant severe acidosis, and/or hyperkalemia)
Seizures	Protect airway Benzodiazepines Other antiseizure medications: Dilantin
Acidosis	Exclude or treat hypoglycemia, hypovolemia, and sepsis If severe, performing hemofiltration or hemodialysis is indicated
Severe anemia	Blood transfusion
Antiparasitic treatment	First-line treatment Second-line treatment of complicated malaria Quinine dihydrochloride combined with either clindamycin (300 mg) or doxycycline (100 mg) tablets Presumptive anti-relapse therapy with primaquine is required to eradicate the liver stage of *P. vivax* (hypnozoite), similar to *P. ovale* in both low-transmission and high-transmission settings

Table 1. Clinical spectrum and management of severe/complicated *P. vivax* malaria.

The spread of drug-resistant *P. vivax* to parts of Indonesia, other parts of Southeast Asia, and South America highlights the urgent need to revisit the spectrum of disease and of the burden of vivax malaria in order to implement control measures and allocate adequate resources against this neglected infectious disease [1, 37].

http://dx.doi.org/10.5772/64974

7. Conclusion

Malaria infection remains as a leading infectious diseases affecting humankind due to its associated large burden of disease. There is a growing recognition that *P. vivax* contributes to a large proportion of this burden. In particular, coinfection of *P. vivax* and *P. falciparum* seems to be synergistic in terms of leading to severe disease, complications, and death among the most vulnerable populations. The spectrum of disease severity associated with *P. vivax* infection ranges from respiratory failure to renal failure. However, the most important marker of disease severity across borders is produced by hematological abnormalities including severe anemia and thrombocytopenia. Further research is warranted to address the large epidemiologic burden of disease associated with *P. vivax* and to identify preventive strategies. From a clinical perspective, there is a need for further clinical studies to identify strategies to optimize the clinical management of vivax malaria.

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Section 2

Basic Science

Chapter 7

The Biology of Malaria Gametocytes

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65464

Abstract

Gametocytes are sexual precursor cells of the malaria parasite that mediate the transmission of the parasite from its mammalian host to the *Anopheles* mosquito. Unlike the asexual blood stages, which are responsible for the clinical outcome of malaria, gametocytes cause no clinical manifestations. However, they are very crucial for the transmission of the disease thus represent key targets for transmission-blocking interventions. Despite their essential role in malaria transmission, only in the last decade gametocytes became a hot topic of research and their biology is not well understood. This chapter provides a detailed review on the biology of the human malaria gametocytes with emphasis on aspects such as gametocyte commitment, gametocyte maturation (gametocytogenesis), gametocyte metabolism and gametogenesis. Proper understanding of these processes will deepen our knowledge on the gametocyte biology and therefore open up more avenues for the development of malaria transmission-blocking intervention strategies.

Keywords: gametocyte, gametocytogenesis, transmission, gametogenesis, sexual reproduction, mosquito

1. Introduction

Gametocytes are specialized sexual precursor cells that mediate the transmission of the malaria parasite from the mammalian host to the mosquito. Once these cells have gained maturity, they are picked up by an *Anopheles* mosquito during a blood meal. In the midgut of the mosquito, they become activated and differentiate into male and female gametes. The male gamete then fertilizes the female gamete resulting in the formation of a zygote. The zygote further develops into a motile ookinete, which penetrates the gut epithelium and subsequently develops into an

oocyst. The oocyst then matures releasing sporozoites, which migrate to the salivary gland of the mosquito. The parasite is transmitted to another mammalian host through an infected mosquito bite [1]. Gametocytes therefore provide a link in the transmission of malaria from the mammalian host to the mosquito, thereby making them prime targets for transmission-blocking intervention strategies. These strategies rely on either vaccines or drugs, which target the gametocyte or the mosquito midgut stages to block malaria transmission from the human to the mosquito, thereby preventing the spread of the disease.

Despite their essential role in maintaining the parasite life cycle, research on gametocytes has been very much left out of the limelight. Several reasons could be accounted for this: first, gametocytes cause no clinical manifestation in their host as opposed to the asexual blood stages that are responsible for the clinical features of malaria. Second, it has been a great challenge to culture gametocytes because of the cost and time-consuming cultivation procedure, and finally, it has been a hurdle to obtain pure gametocytes for molecular and metabolic studies as well as drug screening purposes. However, in the last decade, the rapid emergence of drug-resistant parasites and mosquitoes, together with the absence of a malaria vaccine, has rekindled the interest of researchers on alternative measures aimed at interrupting the parasite life cycle by targeting gametocytes. This interest has led to the improvement of gametocyte cultivation and purification methods, which have enabled a greater understanding of the gametocyte biology.

In this chapter, we focus on key aspects of the gametocyte biology such as gametocyte commitment, maturation (gametocytogenesis), metabolism and gametogenesis. This will deepen our understanding on the biology of gametocytes and thereby open up more avenues for the development of malaria transmission-blocking intervention strategies.

2. Gametocyte commitment

The asexual blood stage parasites produce the clinical form of malaria due to destruction of the erythrocytes, while replicating in the mammalian host. However, these asexual blood stages cannot be transmitted to the mosquito to continue the life cycle of the parasite. For this reason, a subset of the asexual blood stages is able to switch to the sexual pathway, resulting in the formation of the sexual precursor cells, the gametocytes. The process of switching from the asexual blood stage to the gametocyte is referred to as gametocyte commitment.

Commitment to gametocyte development is believed to take place at some point before schizogony, in which each individual schizont produces a progeny of merozoites that develop into either sexual forms or asexual blood stage parasites, which continue the erythrocytic cycle [2, 3] (**Figure 1**). In addition, merozoites released from a single sexually committed schizont can either become male or female gametocytes and the characteristic female-biased sex ratio observed in the malaria parasite is due to the production of a higher percentage of committed female schizonts than their male counterpart [3, 4].

Until recently, gametocyte commitment was mainly assigned to stress factors such as high parasitaemia, anaemia, host immune response or drug treatment, with an overwhelming

amount of studies to justify these facts. A study by Chaubey and colleagues suggested that the malaria parasite reacts to endoplasmic reticulum stress by switching to gametocytes [5]. The treatment of mice with phenylhydrazine, which induces hyper-reticulocyte formation, stimulates gametocyte formation when infected with *P. chabaudi* [6]. Lymphocyte and serum from Gambian children have also been shown to induce *P. falciparum* gametocyte production [7]. Another study showed that *P. falciparum* cultures grown in reticulocyte-rich blood from patients with sickle cell anaemia result in enhanced gametocyte formation as compared to normal blood [8]. Haemolysis of infected erythrocytes also triggers the formation of *P. falciparum* gametocytes [9]. The treatment of the malaria parasite with drugs such as steroid hormones [10], fansidar [11] and chloroquine or sulfadoxine-pyrimethamine [12] also enhances gametocyte production.

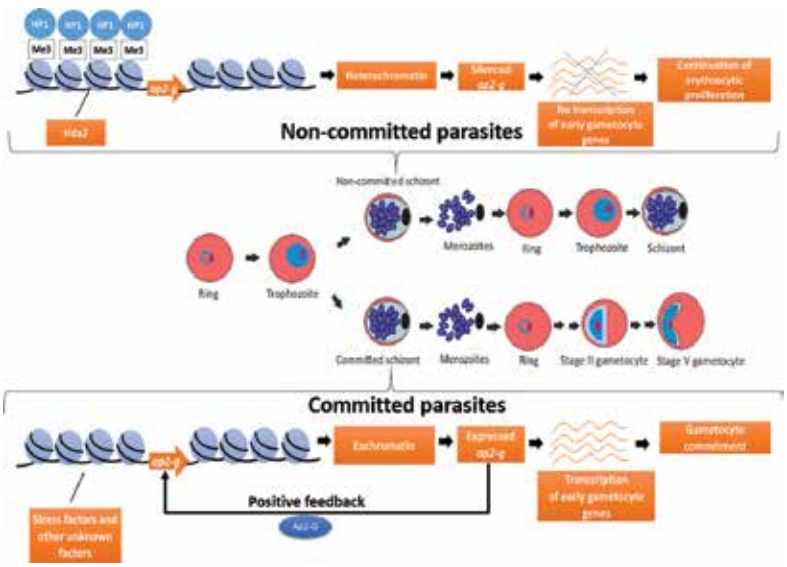

Figure 1. Proposed mechanism of gametocyte commitment. During the erythrocyte cycle, epigenetic and transcriptional regulation work hand in hand to control gametocyte commitment. Two populations of blood stage parasites exist. Non-committed parasites continue the erythrocytic replication of the parasite, and committed parasites are able to differentiate to gametocytes.

However, despite the knowledge that gametocyte commitment can be enhanced by stress factors, the mechanism by which stress induces this process is not well understood. Recent studies have also associated gametocyte commitment to the concerted action of epigenetics and transcriptional regulation.

2.1. Epigenetic mechanism of gametocyte commitment

Epigenetic mechanisms through histone modifications have been shown to regulate gene expression of important proteins in the malaria parasite [13, 14]. In *P. falciparum*, two master players in gene silencing, *P. falciparum* heterochromatin protein 1 (PfHP1) and *P. falciparum*

histone deacetylase 2 (PfHda2) were demonstrated to be implicated in the epigenetic regulation of gametocyte commitment [15, 16].

Heterochromatin protein 1 (HP1) was initially described in *Drosophila* as the main suppressor of position-effect variegation and the major component of heterochromatin gene silencing [17]. Brancucci and colleagues [16] reported that in *P. falciparum*, PfHP1 binds to histone H3-trimethylated residues on lysine 9 (H3K9me3) to maintain heterochromatin gene silencing, which results in the suppression of gametocyte commitment and variegated expression of *var* genes. *Var* genes encode for a family of 60 parasite virulent proteins in *P. falciparum* named *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is present on surface of the *P. falciparum*-infected erythrocyte of which only one is expressed at a time and this phenomenon is associated with antigenic variation. Conditional depletion of PfHP1 results in the hyper-induction of viable gametocytes, thereby demonstrating a role of PfHP1 in suppressing gametocyte commitment. Depletion of PfHP1 also results in derepression of the *ap2-g* gene, which encodes for the AP2-G transcription factor, the only member of the ApiAP2 family shown to associate with PfHP1 [18]. AP2-G was demonstrated to be important in gametocyte commitment in both *P. falciparum* and the rodent malaria parasite *P. berghei* by acting as a transcriptional switch that controls gametocyte differentiation through activating the transcription of early gametocyte genes [19, 20].

Histone deacetylases are histone-modifying enzymes that promote transcriptional silencing by removing acetyl groups from histones. The removal of the acetyl groups facilitates histone methylation and HP1 binding [18, 21, 22], thereby resulting in reduced accessibility for transcriptional factors and heterochromatin formation. A study by Coleman and colleagues [15], which complements the results of Brancucci et al. [16], showed an essential role of PfHda2 in the global silencing of *var* genes as well as controlling gametocyte commitment in *P. falciparum*. Knockdown of PfHda2 results in up-regulation of gene expression of genes associated with gametocytogenesis leading to an increase in gametocyte production. It also results in derepression of the *ap2-g* gene. The authors further reported that most of the genes dysregulated following PfHda2 knockdown were bound to PfHP1, and both proteins localize together with PfHda2-regulated genes, which were significantly enriched in H3K9me3, indicating an interaction between PfHda2 and PfHP1. The two studies therefore provide a link between PfHda2 and PfHP1 in controlling gametocyte commitment.

2.2. Transcriptional regulation of gametocyte commitment

AP2-G is a member of the apicomplexan AP2 DNA-binding protein family, characterized by the presence of an Apetala2/ethylene response factor (AP2/ERF) DNA-binding domain [23]. In the malaria parasite, other AP2 proteins have been assigned to the stage-specific transition during parasite development, for example, the development of midgut, sporozoite and liver stages [24–27].

Two studies in *P. falciparum* and *P. berghei* have shown that AP2-G triggers a transcription cascade that initiates gametocyte commitment [14, 19, 20]. To determine initially, if AP2-G is essential for gametocyte commitment and production, both studies sequenced and analysed the *ap2-g* gene from specific gametocyte-less producing strains for single nucleotide polymor-

phisms, insertions or mutations. Their results showed nonsense or missense mutations in the *ap2-g* gene providing an explanation for the inability of the strains to commit and produce gametocytes. Their initial findings were further confirmed by targeted disruption of the *ap2-g* locus in both *P. falciparum* and *P. berghei*, which resulted in the loss of sexual commitment and gametocyte production. The disruption of the *ap2-g* locus also resulted in the down-regulation of many genes highly expressed in early gametocytes such as *Pfs16* and *Pfg25/27*. The DNA motif involved in AP2-G-binding was identified as GTAC and shown to be located upstream of many genes associated with gametocytogenesis [19, 20, 28]. Because the *ap2-g* gene also possesses the GTAC domain on its promoter region, this suggests that it regulates itself through a feedback mechanism. The AP2-G DNA-binding motif was shown to interact with gametocyte-associated gene promoters in a motif-dependent manner. Mutations of the motif in the promoter region result in a blockage of the gene expression of the gametocyte-associated genes, thereby indicating that AP2-G induces the expression of genes containing the AP2-G DNA motif by binding to it at the promoter region [19].

During the malaria life cycle, therefore, two populations of the malaria parasite exist. Non-committed parasites, which continue the erythrocytic cycle, and committed parasites that form gametocytes. In the case of non-committed parasites, PHP1 binds to H3K9me3 residues thereby maintaining the *ap2-g* locus at a heterochromatic state resulting in silencing of the *ap2-g* expression. This silencing leads to the inability to initiate transcription of genes important for gametocyte commitment; therefore, the parasite continues with erythrocytic replication. PHda2 is also involved in the silencing of the *ap2-g* gene expression in non-committed parasites, probably by the removal of acetylated histone residues allowing for their methylation leading to the binding of PHP1. In committed parasites, stress factors and other unknown factors cause the removal of the histone H3 trimethylation mark, thereby preventing PHP1 from binding to the histone and therefore maintaining the *ap2-g* locus at an active state, resulting in AP2-G expression. The expression of AP2-G promotes the transcription of early gametocyte genes, which leads to gametocyte commitment and formation (**Figure 1**).

3. Gametocyte maturation

3.1. Gametocyte morphology

The morphology of gametocytes varies among the *Plasmodium* species. While mature gametocytes from *P. ovale, P. malariae, P. vivax* and *P. knowlesi* possess a round to oval shape, *P. falciparum* adopts a crescent or falciform shape when matured [29–31].

P. falciparum is named from the crescent or falciform shape of its mature stage. During gametocyte development, the parasite undergoes five distinct morphological stages (I–V) (**Figure 2**). The first observations on the morphology of *P. falciparum* gametocytes were made in the 1950s, when the five development stages were defined [30]. Later studies using light and electron microscopic approaches have defined the shape and time of appearance [32, 33]. Studies have shown that stages I–IV are sequestered into the bone marrow, while stage V circulates in the peripheral blood vessels [34–36]. Stage I gametocyte has a rounded shape,

which is indistinguishable from the young asexual trophozoite. Stage IIa, on the other hand, can be distinguished from the trophozoite by its large round format and granular distribution of pigment in several food vacuoles. In stage IIb, the parasite starts to elongate and a D-shape can be observed. Stage III gametocytes adopt an oval shape with the red blood cell slightly distorted due to further elongated of the parasite. In stage IV, it is possible to distinguish between male and female gametocytes. Both genders have an elongate thin format with pointed tips. In male gametocytes, the pigment tends to be scattered while in female it is denser. The stage V gametocyte is the most distinguishable among all stages due to the crescent or falciform shape. While the male gametocyte is thicker and the cytoplasm appears pale blue following Giemsa staining, the female is more elongate and curved with a blue-stained cytoplasm. This stage is visible in the periphery circulation after 10–12 days of maturation [29].

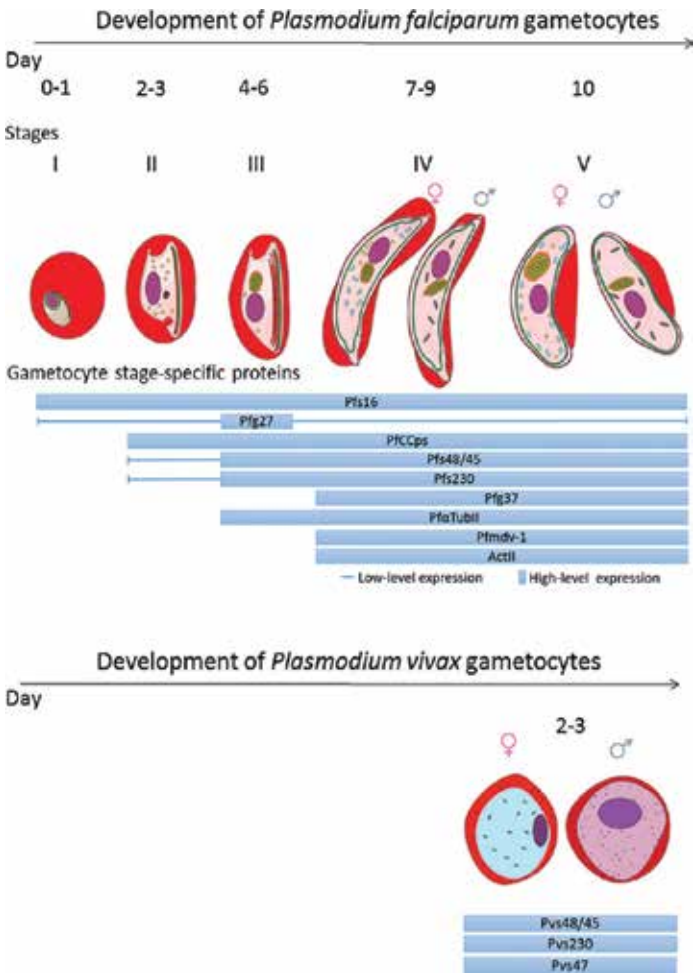

Figure 2. *P. falciparum* and *P. vivax* stage-specific protein expression during gametocyte development. The bar represents high-level expression and full line low-level expression. Only gene products were in included in this figure.

Among the other species of malaria, *P. vivax* is the most important because of its high impact on human health in South and Central America and South Asia [37]. The gametocytes of *P. vivax* are different from *P. falciparum* in their biology, dynamics and their formation, which occur within 2–3 days after asexual blood stage appearance in the circulation [38]. The male and female gametocytes of *P. vivax* possess a round shape as it is observed in *P. malariae, P. ovale* and *P. knowlesi*, but it is only in *P. vivax* that the parasite fills an enlarged red blood cell (**Figure 2**). In Giemsa-stained thin blood smears, female gametocytes have a blue-stained cytoplasm, and the chromatin is a purplish mass with a peripheral distribution, while male gametocytes possess a large and loose chromatin mass.

One remarkable morphological feature of gametocytes is the presence of the inner membrane complex (IMC), which underlies the plasmalemma and consists of flat membranous sacs called alveoli that are coupled to a supporting cytoskeletal network. The IMC was originally assigned to the invasive stages of *Plasmodium* as part of actin-myosin motor complex, and thus, the IMC is important for motility [39]. Recently, it was confirmed that gametocytes also possess an IMC [40–42], which is thought to be required for the stability of the crescent shape. The outer alveolar membrane contains complexes of the gliding-associated proteins GAP40, GAP45 and GAP50, which facilitate binding of myosin to this membrane. The gametocyte alveoli are subtended by a subpellicular network of interwoven alveoli filaments linked to the alveoli via intramembranous particles [39–41, 43]. Underneath the subpellicular network, an array of longitudinally oriented microtubules and F-actin fibrils are found that become reorganized in stage V gametocytes, leading to rounding-up of the gametocyte tips [44].

3.2. Gametocyte-specific proteins

Approximately 20% of all plasmodial genes are specifically expressed in the sexual stages [45, 46]. In *P. falciparum* gametocytes, the proteins are expressed at different levels in the five distinct developmental stages (**Figure 2**). Most of these proteins are associated with gametocyte development, integrity and preparation for fertilization. Furthermore, gender-related proteins are expressed in the later stages in order to support the gametes for fertilization in the mosquito midgut [1, 47–49].

In the early stage I–II gametocytes, important proteins such as Pfs16, Pfg27 and the six LCCL-domain proteins are expressed [50]. Pfs16 is present in all gametocyte stages, but it is initially expressed in stage I. It is localized in the parasitophorous vacuole (PV) where it plays a role in gametocyte development [51–53]. The main function of Pfs16 is unknown, but its disruption leads to a decrease in gametocyte production and an impairment in the ability of microgametocyte to exflagellate [54]. The protein Pfg27 is a phosphoprotein that starts to be expressed from stage II. It is known that Pfg27 forms a homodimer to act as single-stranded RNA-binding proteins *in vitro* and its gene deletion leads to the formation of abnormal gametocytes [55, 56]. The six LCCL-domain proteins (termed CCp in *P. falciparum* and LAP in *P. berghei*) possess adhesive properties and expression begins from stage II. In *P. falciparum* gametocytes, CCp proteins are localized within the PV, but after gamete emergence, the proteins are associated with the surface of gametes [47, 48, 57, 58].

Starting from stage IIb, well-known sexual-stage proteins such as the 6-cys proteins Pfs48/45 and Pfs230 and the osmiophilic body protein Pfg377 and Pfmdv-1 or sexual stage-specific cytoskeletal proteins such as α-tubII (tubulin II) and actin II are synthesized [50]. Similar to the six LCCL-domain proteins, the surface proteins Pfs48/45 and Pfs230 have adhesive properties and gene disruption of both proteins impair male gamete fertility [59, 60]. Pfg377 is known to be associated with osmiophilic bodies in female gametocyte of *P. falciparum*, and gene disruption demonstrates a fundamental role in the formation of osmiophilic bodies [61, 62]. The Pfmdv-1 protein, also known as Pfpeg3, is highly expressed in early gametocyte until stage V afterwards reaching a low level of expression. The function of *Pfmdv-1* is still unknown although disruption of the *Pfmdv-1* encoding gene leads to a 20-fold decrease in the production of male gametocytes [63]. The cytoskeletal proteins α-tubulin II and actin II are sexual stage-specific, and both are shown to be predominantly present in male gametocytes [64–66]. While α-tubulin II has been described to incorporate to axonemes of emerging male gametes, the function of actin II is still unclear, but gene disruption of both proteins leads to an impairment of male gametogenesis [65, 66]. In the mature microgametocyte, the protein PfB0400w, also known as PfMR5, can be observed. Although its function is still unknown, the expression of PfB0400w in male stage V gametocyte indicates that it may play a role in preparing gametocytes for exflagellation [50, 67].

In the gametocytes of *P. vivax*, three sexual stage-specific proteins have hitherto been identified, i.e. the 6-cys proteins Pvs230, Pvs48/45 and Pvs47, all of which are orthologues of those present in *P. falciparum* (**Figure 2**). Pvs230 has been characterized as an antigen on the surface of gametocytes and gametes while Pvs48/45 and Pvs47 have been studied recently as potential candidates for transmission-blocking vaccines. Following direct membrane feeding assays, anti-Pvs48/45 and anti-Pvs47 antibodies significantly reduced oocyst numbers in the mosquito midgut [68–70].

4. The gametocyte metabolism

During gametocytogenesis, *P. falciparum* parasites undergo intense metabolic processes including energy production as well as synthesis and degradation of various molecules such as lipids and proteins. These processes are essentials for the survival of the malaria parasite and ensure gametocyte stage development, drug resistance and transmission to the mosquito vector [30, 71, 72].

As vital molecules, lipids are responsible for many functions such as cell signalling, energy storage and the stability of membranes. Furthermore, during the various stages of the *Plasmodium* life cycle, lipids play a crucial role in the growth and proliferation of the parasite [73]. Among asexual blood stages and gametocytes, there are more than 300 lipids with known and unknown functions [74]. Although not reflected in all classes of lipids, the amount of lipids would more than double during gametocytogenesis [75]. The lipid composition of all developmental stages differs, including during gametocyte maturation. It is known that sphingolipids involved in ceramide metabolism are enriched during gametocyte maturation and the

inhibition of the ceramide biosynthetic pathway might kill gametocytes. In addition, phosphatidylserine is also highly expressed during gametocytogenesis and together with ceramides leads to gametocyte induction [74]. Neutral lipids such as cholesteryl ester and diacylglycerol (DAG) have also been shown to be significantly increased during gametocyte maturation; however, more data to better understand the metabolism and function of these lipids during gametocytogenesis are required [75].

In gametocytes, no DNA-replication occurs [76] and the nucleic acid synthesis is exclusive for RNA production. The production of RNA ceases from around day 6 of gametocytogenesis, thus the RNA and protein synthesis are more important in the early than late gametocyte stages [77, 78]. One interesting feature of malaria parasites is the expression of small subunit ribosomal RNA (SSU rRNA) genes [79], seven of them are synthesized in *P. falciparum* and one (S-type, formally known as C-type) is exclusively expressed in gametocytes. Proteomics identified 931 different proteins (72.2% of the proteins identified in *P. falciparum*) in gametocytes and within these proteins include conserved, stage-specific, secreted and membrane-associated proteins [46]. It is also known that protein expression differs between gender, female gametocytes synthetize proteins required for mitochondria and ribosomes, while male gametocytes only need proteins for DNA synthesis, axoneme formation and exflagellation [29, 80].

In eukaryotes, the mitochondria have many vital roles such as ATP synthesis, calcium homeostasis, lipid metabolism and synthesis of ion-sulphate clusters. The malaria parasites only possess a single mitochondrion and during parasite development it is observed that the mitochondria of gametocytes are longer and possess more cristae when compare to the ones of the asexual blood stages. This suggests that ATP synthesis via the electron transport chain is the main mechanism of energy production in gametocytes [29, 33]. Indeed the sexual stages appear to have seven times more cytochrome b than the asexual blood stages [81]. The cytochrome bc1 complex feeds the electron transport chain with more protons, which creates a gradient that drives ATP synthesis [82]. The cytochrome bc1 is a prime target for the antimalarial drug atovaquone that inhibits ubiquinone leading to the inhibition of *Plasmodium* cytochrome bc1 activity and loss of mitochondrial function [83–85]. Other drugs such as primaquine and the primaquine analogue bulaquine have been shown to exhibit gametocytocidal activities and primaquine is suggested to have the site of action in the mitochondria by causing its deformation. Although the mode of action is poorly understood, these drugs reduce gametocytemia in infection by *P. falciparum* and have an effect against the exo-erythrocytic stages of *P. vivax* [86].

Despite the fact that the mitochondria have been well characterized in other organisms, many mitochondrion-associated events in *P. falciparum* are still not well understood. Initially, *P. falciparum* blood stage parasites were thought to be glucose- and glycolysis-dependent for carbon metabolism, ATP production and survival [87]. However, recent reports have also shown the importance of the tricarboxylic acid (TCA) cycle for energy production, especially in gametocytes [88]. The gametocyte utilizes mitochondrial TCA to catabolize host glucose and glutamine. The carbon skeleton derived from these molecules enters in the TCA cycle via acetyl-CoA to generate proton flux and ATP via the ATP synthase. Due to the role of the TCA

cycle in parasite development, inhibition of this metabolic pathway leads to defects in gametocytogenesis. In addition, many mitochondrial enzymes involved in metabolic pathways can be targets for drug development [88].

5. Gametocyte activation and gametogenesis

5.1. The signalling cascade of gametocyte activation

The mature stage V gametocytes, which circulate in the blood of the mammalian host, are picked up by the female *Anopheles* mosquito during a bite and enter the midgut together with the blood meal. During the process, the gametocytes receive environmental signals, which indicate the switch from the warm-blooded host to the insect vector. The perception of these signals results in the immediate activation of the gametocytes followed by the onset of gametogenesis. Signals inducing gamete formation include a decrease in temperature by approximately 5°C, which is mandatory for gametocyte activation, and the presence of the mosquito-derived molecule xanthurenic acid (XA), a by-product of eye pigment synthesis [89–91].

While the plasmodial receptor or receptors important for signal perception are still unknown, the signalling pathway resulting in gametogenesis has partially been deciphered (**Figure 3**). It has previously been shown that male gametogenesis, during which the activated male microgametocyte forms eight motile flagellar microgametes (a process termed exflagellation), involves a fast increase in intracellular calcium and the second messenger cGMP [92, 93]. Two membrane-integrated guanylyl cyclases (GCα and GCβ) were identified in *Plasmodium*; however, only GCα appears to be important for cGMP synthesis in activated gametocytes [94–97]. The guanylyl cyclase can be stimulated by addition of XA. It has to be clarified, though, if XA acts directly or indirectly on GCα.

While in eukaryotes, cGMP is synthesized by guanylyl cyclases, phosphodiesterases (PDEs), which hydrolyse cGMP to GMP, are in general required for fast down-regulation of the second messenger. In *Plasmodium*, four PDEs, termed PDEα-δ, were identified, and PDEδ is highly expressed in gametocytes [98]. Zaprinast, a PDE inhibitor, is able to stimulate gametogenesis in the absence of XA due to increased cGMP levels [99]. Disruption of the gene encoding for PDEδ, however, impaired egress of *P. falciparum* gametocytes, indicating that a tight regulation of cGMP levels is important for gametogenesis [97].

The increase in cGMP activates the cGMP-dependent protein kinase PKG and chemical inhibition of PKG impairs rounding-up of the activated gametocytes. Since rounding-up is a calcium-independent step and cannot be inhibited by calcium chelators (see below), PKG acts prior to calcium increase in the activated gametocytes [97]. Gametocyte activation further leads to the generation of the second messenger molecules DAG and inositol-(1,4,5)-trisphosphate (IP_3) from phosphatidylinositol-(4,5)-bisphosphate (PIP_2) via the plasmodial phospholipase PI-PLC activity (**Figure 3**), as shown in *P. berghei*. IP_3 synthesis results in a rapid mobilization of calcium from the endoplasmic reticulum [100, 101]. PI-PLC activity itself appears to be

regulated by calcium, since it can be impaired by calcium ionophores, pointing to a calcium-regulated feedback mechanism. The increase in cytoplasmic calcium levels is further dependent on PKG activity and quantitative phosphoproteomics demonstrated that PKG is able to phosphorylate enzymes involved in the inositol phospholipid metabolism such as phosphatidylinositol-4-kinase (PI4K) and phosphoatidlyinositol-4-phosphate-5-kinase (PIP5K), which are involved in the synthesis of PIP_2 from phosphatidyl-1D-*myo*-inositol (PI) via phosphatidylinositol-4-phosphate (PI4P) [102].

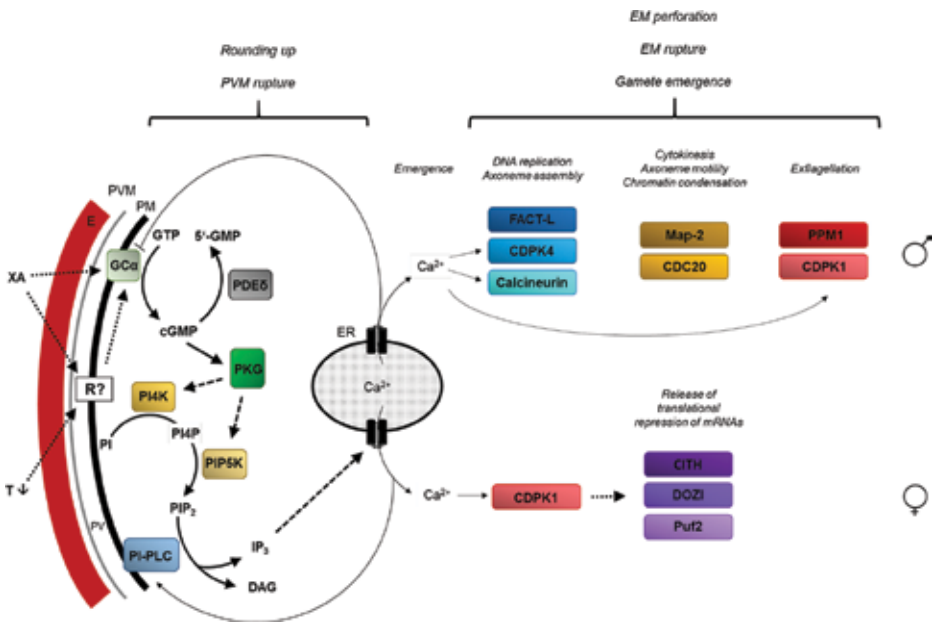

Figure 3. The signalling pathway involved in gametogenesis. Ca^{2+}, calcium ion; CDC20, cell division cycle protein 20; CDPK, calcium-dependent protein kinase; cGMP, cyclic guanosine monophosphate; CITH, homolog of worm CAR-I and fly Trailer Hitch; DAG, diacylglycerol; DNA, deoxyribonucleic acid; DOZI, development of zygote inhibited; E, erythrocyte; EM, erythrocyte membrane; ER endoplasmic reticulum; FACT-L, facilitates chromatin transcription protein L; GC, guanylyl cyclase; 5′-GMP, guanosine monophosphate; GTP, guanosine triphosphate; IP_3, inositol triphosphate; Map-2, mitogen-activated protein kinase 2; mRNA, messenger ribonucleic acid; PDE, phosphodiesterase; PI, phosphatidyl-1D-*myo*-inositol; PI4K, phosphatidylinositol-4-kinase; PI4P, phosphatidylinosito-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP5K, phosphatidylinositol-5-kinase; PKG, cGMP-dependent protein kinase; PPM1, metallo-dependent protein phosphatase 1; Puf2, pumilio/FBF (fem-3 binding factor) family protein 2; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; R, receptor; T, temperature; XA, xanthurenic acid.

In activated *P. berghei* microgametocytes, the increase in intracellular calcium was shown to activate the calcium-dependent protein kinases CDPK1 and CDPK4 and parasites lacking CDPK4 are not able to undergo DNA synthesis [103] (**Figure 3**). Further linked to this process is plasmodial FACT-L (facilitates chromatin transcription) [104]. Downstream of DNA replication two more proteins become activated, the mitogen-activated protein kinase MAP2 and the cell division cycle protein CDC20. Loss of MAP2 or CDC20 interferes with chromatin condensation, axoneme motility and cytokinesis [105–107]. Furthermore, depletion of the metallo-dependent protein phosphatase PPM1 or the calcium-dependent phosphatase

calcineurin as well as knock-down of CDPK1 result in impaired exflagellation [108–110]. While these combined studies pinpoint some key regulators of gametogenesis, the detailed pathway leading to gametocyte-to-gamete conversion is not known yet.

In female macrogametocytes, activation results in a sudden onset of protein synthesis, which is due to the CDPK1-regulated release of translational repression of messenger RNAs (mRNAs) [108]. In *P. berghei*, a total of 731 mRNAs are associated with two proteins of regulatory ribonucleoprotein complexes, i.e. the RNA helicase DOZI (development of zygote inhibited) and the Sm-like factor CITH (homolog of worm CAR-I and fly Trailer Hitch), which function in translational repression of these transcripts (**Figure 3**). These include the mRNAs encoding for P25, GAP45 and GAP50 [111–113]. For *P. falciparum*, translational repression of mRNAs was shown to involve the Pumilio/FBF (Puf) family RNA-binding protein Puf2 that is required for controlling a number of transcripts in gametocytes including those encoding for P25 and P28 [114]. The repressed mRNAs mostly encode for components of the later ookinete stage.

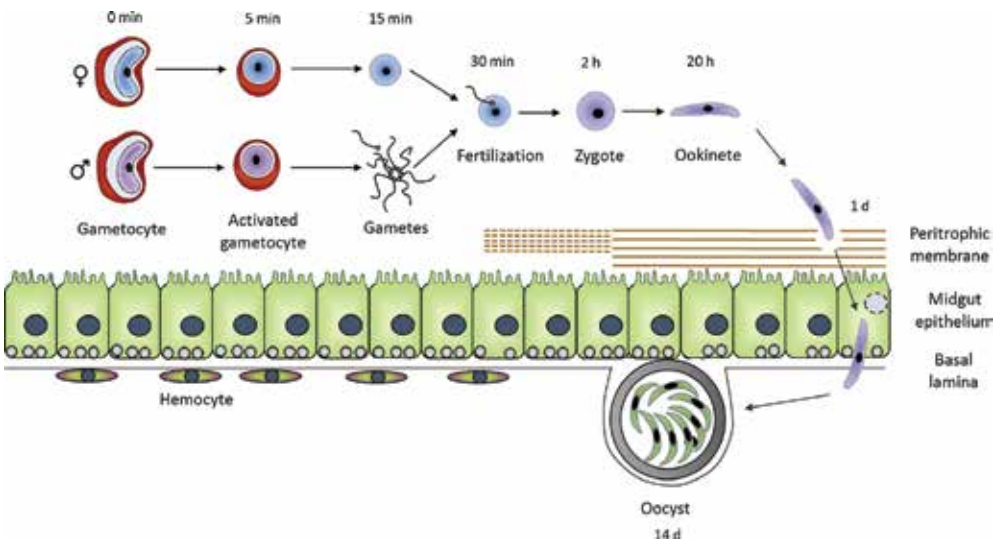

Figure 4. Development of malaria parasites in the mosquito midgut.

5.2. Gametocyte egress and gamete formation

Gametocyte activation results in three morphological changes in the parasites, i.e. rounding-up, erythrocyte egress and gamete formation, and these changes occur with approximately 15 min following perception of the environmental signals (**Figure 4**). Gametocytes exit the erythrocyte via an inside-out mode, during which the parasitophorous vacuolar membrane (PVM) ruptures prior to the opening of the erythrocyte membrane (EM). Rupture of the PVM occurs at multiple sites less than 2 min following signal perception and is accompanied by the rounding-up of the parasite. Both events are calcium-independent [91, 99]. It is suggested that

the PVM rupture is linked to the discharge of the osmiophilic body content into the PV, since an accumulation of osmiophilic bodies can be observed underneath the rupture sites [91, 115].

In a second, a calcium-dependent step, another type of vesicle discharges its content into the cytoplasm of the host erythrocyte, which includes the plasmodial pore-forming perforin PPLP2. This perforin is able to permeate the EM, resulting in the release the erythrocyte cytoplasm, eventually leaving the forming gamete enclosed by the EM [115–117]. While EM permeabilization occurs approximately 6 min post-activation, its rupture takes place 9 min later. Here, the EM opens at a single pore and releases the fertile gamete [91, 117].

The IMC disintegrates shortly after onset of gametogenesis, eventually resulting in gametes solely confined by the plasmalemma. It was shown that the transmembrane protein GAP50, which in gametocytes, is located in the outer membrane of the alveoli, relocates to the gamete plasmalemma during gametogenesis. During relocation, the N-terminal part of GAP50 becomes extracellularly exposed and once the gametes have freed from the EM binds complement factor H from the blood meal, a step crucial to protect the newly formed gametes from lysis by human complement factors [42]. Coevally with the IMC degradation long membranous tubules, termed tunneling nanotubes (TNTs), form, which protrude from the gamete surface. TNTs represent long-distance cell-to-cell connections, which were proposed to mediate intercellular contacts between gametes as a pre-requisite for mating [118]. While the question of the origin of the membranous tubules remains unanswered, one might stipulate that after IMC disintegration the alveoli membranes are used for TNT formation.

Upon activation, the male microgametocyte replicates its genome three times and mitotically produces eight flagellar microgametes [119] (**Figure 4**). Flagellum formation requires the axonemal assembly from basal bodies, which involves the centriole/basal body protein SAS-6 [120], while axoneme motility is regulated by the conserved armadillo-repeat protein PF16 [121]. Exflagellating microgametes typically adhere avidly to neighbouring erythrocytes and are hidden within such rosettes. During exflagellation, the microgamete detaches from the residual body and is freely motile in search of a female macrogamete. Once adhering to a macrogamete, fertilization begins by the fusion of the two gamete plasma membranes, and the axoneme and attached male nucleus enter the female cytoplasm. While the exact proteins involved in initial binding of male and female gametes are yet unknown, gamete fusion is mediated by the microgamete-specific protein GCS1 (generative cell specific 1), also termed HAP2 [122, 123]. Over the next 3 hours, meiosis occurs and the zygote becomes tetraploid [119]. The transformation of the zygote into an ookinete is completed at 19–36 hours post-blood meal, and the ookinete then rapidly exits the midgut lumen to settle down at the basal site of the midgut epithelium and to differentiate into an oocyst [124–127]. Parasite tetraploidy persists until sporozoite budding in the oocyst restores the haploid state [119].

6. Conclusion

Although our knowledge of the biology of gametocytes is not as advanced as that of the asexual blood stages, the great interest by researchers in recent years has considerably deepened our

understanding of the gametocyte biology. Advances in gametocyte cultivation, isolation and purification techniques as well as the impressive amount of data gained from genome-wide analyses, proteomics and microarray studies have led to the identification of some gametocyte stage-specific antigens as well as sex-specific proteins, which may be exploited as targets for malaria transmission-blocking intervention strategies. In addition, the recent discoveries on the molecular mechanism of gametocyte commitment have greatly improved our knowledge on how sexual differentiation is initiated and regulated in the malaria parasite. However, several questions are still to be answered regarding the biology of gametocytes. The mechanism by which stress factors are perceived to enhance gametocyte commitment is not well understood. Some components of the signalling cascade of gametocyte activation are yet unknown such as the receptor or receptors responsible for signal perception following gametocyte activation by XA. How gametocyte sequestration in the bone marrow takes place as well as the molecular mechanism underlying this phenomenon has to been deciphered. Also, a proper understanding of the mechanism by which the gametocyte rapidly adapts once in the mosquito vector and the basis of fertilization is needed. Furthermore, not much is known about the metabolism of gametocytes. Most of the metabolic pathways have not been described. A proper understanding of these aspects in the biology of gametocytes would greatly improve strategies aimed to target gametocytes.

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Chapter 8

Structure and Functional Differentiation of PfCRT Mutation in Chloroquine Resistance (CQR) in *Plasmodium falciparum* Malaria

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64659

Abstract

Approximately one million deaths are attributed to malaria every year. Latest reports of multi-drug treatment failure of falciparum malaria underscore the desideratum to understand the molecular substratum of drug resistance. The mutations in the digestive vacuole transmembrane protein *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) are mainly responsible for chloroquine resistance (CQR) in *Plasmodium falciparum*. Multiple mutations in the PfCRT are concerned in chloroquine resistance, but the evolution of intricate haplotypes is not yet well understood. *P. falciparum* resistance to chloroquine is the standard antimalarial drug and is mediated primarily by mutant forms of the PfCRT. In this chapter, we present the mechanism of action of the chloroquine, the structural changes of the gene after the mutations as well as different haplotypes of the PfCRT.

Keywords: antimalarial resistance, haplotype, homology modeling, mutations, PfCRT

1. Introduction

The rapid advancement and spread of malaria parasite along with antimalarial resistance is becoming a critical disaster to the world health. Chloroquine resistance (CQR) originated in Southeast Asia and South America, more or less simultaneously, in late 1950s and subsequently spread to several other malaria-endemic countries [1]. PfCRT, a candidate gene for

CQR, is present at the digestive vacuole membrane and it holds 10 putative transmembrane domains [2, 3]. Mutations in two genes, namely, the *Plasmodium falciparum* CQ resistance transporter (PfCRT) and multidrug resistance transporter-1 (pfmdr1), have been reported as responsible for CQR in *P. falciparum*. In addition, the polymorphisms of the PfCRT gene produce two different forms of PfCRT based on the drug response class, such as Chloroquine Sensitive (CQS) and CQR. The point mutations of the PfCRT codons are 72–76, 271, 326, 356, and 371, whereas two codons responsible for pfmdr1 are 86 and 1246 as molecular markers of CQ resistance [4]. The position of 72–76 of the PfCRT are considered as molecular markers used for detecting CQR malaria parasites due to the mutations in the positions 72–76 of the PfCRT, which were observed as a majority of *P. falciparum* endemic areas. There are five polymorphisms that form different haplotypes vary among the *P. falciparum* endemic regions. There are three major haplotypes based on specific mutations such as CVIETIHSESI, CVIETIHSESTI, and SVMNTIHSQDLR [2, 5–7].

Chloroquine was used as a synthetic drug in the early 1950s and 1970s [8]. The resistance to this drug was reported in Palian area of Cambodia nearer to Thai-Cambodia border as well as in Latin America. Subsequently the reported resistance to chloroquine in South Asia moved westward and during 1973, it was found to be present in North East region of India. Subsequently it spread to rest of India and beyond [9]. In 1950s, before the chloroquine resistance became widespread, it was the main drug which was cheap and having least toxicity as well as highly effective schizonticidal drug and also was effective against all the types of parasite species affecting human. But appearance of widespread resistance to chloroquine has contributed to resurgence of malaria in many countries of Asia including India [10].

By late 1980s, chloroquine became more or less obsolete for treating *P. falciparum* infections globally. However, due to high economical burden in introducing alternate treatment such as artesunate combination therapy, etc. for treatment of *Plasmodium vivax* restricted many countries like India to continue only with chloroquine for treatment of *P. vivax* [11]. This dual drug policy for the treatment of *P. falciparum* and *P. vivax* resulted in further consolidation of *P. falciparum*-resistant population in various southeastern regions of the Asia, especially in India [10].

In this chapter, we estimated certain scores of mutations such as (limbo, tango, and waltz score) to understand the changes that occur to the PfCRT protein after mutation with the help of homology modeling and single-nucleotide polymorphism (SNP).

2. *P. falciparum* CRT as a target for antimalarial drug design

Earlier, the parasite proteins involved in the resistance mechanism of malaria were unknown, but currently, it is well understood that the mutations in the PfCRT gene are causally involved in various methods such as *in vitro* and *in vivo* resistance as well as altered drug accumulation [2, 3, 12]. Identification of PfCRT gene, which encodes a putative transporter or channel protein, was a major achievement in the search for the genetic basis of CQR in *P. falciparum* [2]. PfCRT is a 48-kDa protein having 424 amino acids, 13 exon gene spanning 36 kb of chromosome 7

http://dx.doi.org/10.5772/64659

and 10 predicted transmembrane-spanning domains and is confined in a small area to the Digestive Vacuole (DV) membrane in erythrocytic stage parasites [2, 3]. The polymorphisms of PfCRT segregate precisely with two distinct drug response, which is considered either CQS or CQR. Fifteen polymorphic amino acid positions in PfCRT are associated with CQR in field isolates. These vary significantly depending on the geographic location and selection history, while CQS strains maintain an invariable wild-type allele [5–6, 13, 14]. K76T and S163R mutations in $PfCRT^{CQR}$ are primary and necessary for the resistance phenotype, which is the most reliable molecular marker of resistance among the various PfCRT mutations [10, 15, 16]. The endogenous role of PfCRT in the malaria parasite has not been clear yet despite the wealth of epidemiological and *in vitro* drug response data demonstrating the critical role of PfCRT mutations in producing CQR. An understanding of the natural role of PfCRT in a normally functioning cell is indeed needed to provide a clearer picture of how drug resistance works in the malaria parasite. Muhamad et al. studied the polymorphic pattern of PfCRT, which may be applied surveillance of chloroquine [17].

3. Mechanism of PfCRT

The mechanism involved in resistance against quinoline containing compound CQ in *P. falciparum* is still unclear [18, 19] and indeed the mode of action of this antimalarial chemotherapeutic agent is not beyond the debate. Chloroquine is thought to accumulate in high levels in the food vacuole of the asexual erythrocytic malaria parasites, where it acts by interfering with the polymerization of heme (hematin) into the hemozoin. The malaria parasite feeds by degrading hemoglobin of host cell, producing free ferriprotoporphyrin IX (FP) as a by-product. Ferriprotoporphyrin IX (FP) is highly toxic to the parasite and is neutralized via a process of biomineralization (polymerization) to form innocuous hemozoin crystals (β-hematin) known as malaria pigment. Chloroquine is thought to inhibit parasite growth by inhibiting the detoxification of ferriprotoporphyrin IX (FP) [20–23]. Chloroquine (CQ) appears to trap FP in a μ-oxodimeric form and prevents the formation of the β-hematin dimers that are required for hemozoin formation [24]. Thus, CQ causes a buildup of toxic FP molecules that eventually destroy the integrity of malaria parasite protein and membranes [25].

Current studies highlight an important gene connected to the resistance, *P. falciparum* chloroquine resistance transporter (PfCRT). It encodes a new transporter and also differentiates between the global selective sweeps of different haplotypes having the mutation, K76T [26]. The PfCRT gene which produces the transport proteins on the plasma membrane of the parasite's food vacuole, have been confirmed of involving with the resistance of the parasite to antimalarial drugs [27].

The mechanisms involved in the development of CQ resistance are also unclear. It is postulated that CQ resistance could arise as a consequence of any phenotypic and genotypic alteration(s), which reduces the concentration of the drug in the food vacuole of the parasite. This leads to a change in parasite biology and can lead to reduced uptake of the drug or enhanced CQ efflux from the cell or a combination of both resulting in reduced accumulation of drug inside the

digestive vacuole of the parasite. It has been established that in CQ-resistant parasites, the accumulation of chloroquine inside the vacuole is significantly less than that in CQ-sensitive parasites [28, 29]. It was originally thought that this lack of accumulation was due to the result of an efflux mechanism, and P-glycoprotein was implicated as the pump responsible for the efflux. However subsequent studies have suggested that efflux rates of CQ-resistant and CQ-sensitive strains are similar. So it appears that CQ resistance involves a diminished level of drug uptake rather than, or as well as, enhanced efflux. Chloroquine-resistant parasites are known to get rid themselves of the drugs 40–50 times faster than sensitive parasites [30] but the biochemical basis of this efflux is not clear. The efflux of chloroquine and in fact the entire chloroquine-resistant phenotype can be reversed with Ca^{+} channel blockers such as verapamil and diltiazem [31]. This phenomenon is biologically very similar to multi-drug resistance (MDR) phenotype of mammalian tumor cells, where a wide spectrum of chemotherapeutic agents is expelled from the cells by a verapamil-sensitive pump [32–35]. Verapamil (VPL), which inhibits P-glycoprotein (encoded by mdr gene) mediated multi-drug resistance (MDR) in mammalian tumor cells, also partly reverses chloroquine resistance in malaria parasites grown *in vitro* [31]. Because this reversal phenomenon is in some ways analogous to the reversal of MDR in mammalian tumor cell lines, CQ resistance has been postulated to involve an energy driven P-glycoprotein pump similar to that encoded by the mammalian mdr gene [31]. Two *P. falciparum* genes, which are homolog to mammalian mdr, have been identified and mapped to chromosome 5 and named pfmdr1 and pfmdr2 [36–38]. Associations have been reported between chloroquine resistance and mutations or amplification of the mdr-like gene pfmdr1, which encodes P-glycoprotein homolog-1 (Pgh1) [36, 39], which has been found to be located in the food vacuole membrane of the erythrocytic stages of the parasite [40], suggesting that it could be involved in drug transport across this membrane [41]. DNA sequencing of the pfmdr1 gene from reference strains and field isolates has revealed several point mutations that correlated with CQ resistance [39]. In earlier studies, several point mutations or nucleotide changes at position 754, 1049, 3598, 3622, and 4234 in pfmdr1 gene resulting in amino acid change at codons 86, 184, 1034, 1042, and 1246, respectively, have been shown to be associated with CQ resistance [42, 43]. But the role of these mutations in pfmdr1 in CQ resistance is controversial [44–51]. It has been observed that most of the southeast Asian CQ-resistant isolates (K1 genotype, on the basis of 3′ polymorphism in pfmdr1) have the mutation at nucleotide 754 resulting in amino acid change at codon 86 from asparagines to tyrosine (N86Y) while CQ-resistant South American isolates (7G8 genotype based on 3′ polymorphism in pfmdr1) have not shown mutation at codon 86 but mutational change at codon 184, 1034, 1042, and 1246 have been shown. Out of these several mutations described, the mutation at codon 86 appears to be an important, as this may be involved in substrate specificity of the gene product (P-glycoprotein) and may alter the transport activity of the protein [52]. Moreover, mutation at codon 86 has also been correlated to CQ resistance in parasites selected *in vitro* for CQ resistance [53]. However, studies from different geographical areas of the world have given the controversial picture of this mutation. Some field studies have confirmed the presence of this mutation in chloroquine-resistant isolates from Malaysia [42], Nigeria [54] Guinea-Bissau [55], and sub-Saharan Africa [56]. No association with this mutation, however, was found in isolates from Sudan [44], Thailand [45], and Cambodia [46]. Moreover, genetic studies found

http://dx.doi.org/10.5772/64659

no linkage between chloroquine resistance and the pfmdr1 gene [57]. In India, Bhattacharya and Pillai have found strong association between chloroquine resistance and certain mutations in the pfmdr1 gene of *P. falciparum* isolates [58]. However, that study was conducted in small number *P. falciparum* strains. Gómez-Saladín et al. found the involvement of tyr86 mutation of pfmdr1 gene in CQ resistance but no significant association between tyr86 mutation and level of resistance with chloroquine [59]. Majority of pfmdr1 studies reported till date have indicated conflicting pieces of evidence, which suggest that CQ resistance in *P. falciparum* involves multiple transport mechanisms and multiple genes.

Another locus governing chloroquine resistance in a *P. falciparum* genetic cross has been mapped on chromosome 7 and has been named as cg2 (candidate gene) [57, 60]. A protein specified by this gene has size variation in three repeat regions (k, ϕ, and ω) several non-silent point mutations and size variation in a central poly-Asn tract. Recently, field studies have identified a series of mutations in cg2 gene in chloroquine-resistant *P. falciparum* strains suggesting that polymorphism in cg2 gene was highly associated with CQ resistance [61, 62]. Chloroquine-resistant strains have been found to consist of 16 tandem repeat units in omega repeat region (Dd2 type) of cg2 gene, while the chloroquine-sensitive strains have either ≤15 or ≥17 repeat units [54]. A significant but incomplete association has been found between the presence of the cg2 Dd2-like omega repeat length polymorphism and *in vitro* resistance and between the tyr86 allele of pfmdr1 and *in vitro* resistance [49]. Adagu and Warhurst have reported the ala281 mutation in cg2 gene and Dd2-type kappa (k) repeats in CQ-resistant isolates of northern Nigeria [62]. They have also reported the significant association between tyr86 mutation in pfmdr1 and Dd2-type kappa (k) repeat and ala 281 mutation of cg2 gene.

Recently PfCRT, a gene with 13 exons, has been identified near cg2 on chromosome 7 [2]. This gene encodes, a transmembrane protein "PfCRT" in the digestive vacuole of malaria parasites. Sets of point mutations in PfCRT were associated with *in vitro* chloroquine resistance in clinical isolates and laboratory lines of *P. falciparum* from Africa, South America, and southeast Asia [7, 12, 14, 63–66]. One mutation, the substitution of lysine (K76) by threonine (T76) at position 76 (K76T), was present in all resistant isolates and absent in all sensitive isolates tested *in vitro* [12, 14, 64, 66–69]. A significant association was found between an allele of the *P. falciparum* chloroquine resistance transporter gene (PfCRT-T76) with both *in vitro* and *in vivo* resistance and a significant association between pfmdr1-tyr86 and PfCRT-T76 has also been observed among resistant isolates, which suggests a joint action of the two genes in high-level CQ resistance [65]. Furthermore, it has also been observed that although the PfCRT-K76T mutation was present in all CQ-resistant isolates, yet this mutation was also present in patients who recovered clinically after CQ therapy [68, 70]. Hence, it has been suggested that other mechanism(s) might be involved in modulating outcome of therapy in such cases [64].

The nature of these genetic polymorphisms and their relationship with drug-resistant strains has not been studied in Indian strains of *P. falciparum*. Further, a specific marker capable of diagnosing a chloroquine resistance is yet not available. It was therefore, important to investigate whether the CQ-resistant *P. falciparum* parasites could be identified directly from a blood sample by a rapid, sensitive, and specific method like PCR, which may be of great help in prompt treatment of falciparum malaria, particularly the severe and complicated one.

Therefore, the present study was undertaken to investigate the mutation(s) and genetic polymorphism(s) in the gene PfCRT.

There is a divergence in the literature for the exact mechanism of PfCRT-producing CQR. One theory is that by using energy to transport CQ out of the DV, the protein mediates active drug efflux (similar to that of HuMDR1), which makes it away of its targets [71]. Mutations in the protein may alter its substrate specificity by using this model, which leads to greater CQ affinity for mutant isoforms.

One more hypothesis is that PfCRT facilitates the diffusion of the charged drug species (which is also known as "charged drug leak" hypothesis; [72, 73]). Inside the acidic DV, the drug molecules present are charged as compared to the outside of the DV, which neglects the drug binding. The charged molecules require some kind of carrier as they cannot pass through the hydrophobic environment of a membrane. A benefit of this suggestion is that it provides an explanation for the significance of the K76T mutation. In this mutation, the lysine in wild-type CQS isoforms has a basic side group having positive charge that repels protonated CQ, while the neutral threonine allows for an open pore through which charged CQ may pass.

Another mechanism is there based on the pH alterations of the DV, which may be influenced by PfCRT. It has been shown that CQR parasites have a more acidic DV than CQS parasites by calculating the pH of the DV [74], which is very surprising because at low pH weak base partitioning would predict increased drug accumulation. However, the rates of hematin aggregation and hemozoin formation are increased at acidic pH; as a result, it would reduce the quantity of target available for CQ binding [2]. The amount of surplus unbound drug may alter the equilibrium of passive drug accumulation [73] or may be transported out of the DV by mutant PfCRT [75].

4. Structure of *P. falciparum* CRT and its mutants

The PfCRT gene identified as the determinant gene for CQR gene since the genetic cross between a CQR clone of Indochina (Dd2) and a CQS clone of Honduras (HB3) [57]. The PfCRT protein is of 48.6 kDa, which contains 424 amino acids encoded by a 13 exon gene in the chromosome having 36-kb segment (**Figure 1**) [2]. It may catalyze chloroquine quinine flux

Figure 1. The homology model of the protein using the genebank sequence.

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with H^+ across the digestive vacuole membrane having with 10 putative TMSs [76]. Nessler et al. observed activate various endogenous transporters in frog oocytes, which helps in transporting quinoline drugs including quinine and quinidine [77]. The drug specificity that determines levels of accumulation is because of the mutations in TMSs 1, 4, and 9 alter, which builds an idea of these TMSs play a role in substrate binding [78]. The substrates responsible for PfCRT mutants are chloroquine-resistant reversers [79].

Rapid progressions of chloroquine resistance (CQR) have activated the identification of some other genetic target(s) in genome of *P. falciparum* such as the mutation in K76T of PfCRT gene including three other positions 72, 74, and 75 [80]. The three mutations may present high resistance to CQ than the K76T mutant [81].

The protein is a member of the drug metabolite transporter (DMT) superfamily (TC #2.A.7) [82]. PfCRT contains drug/metabolite transporter domain. This domain is found in protein which is engaged in pectinase, cellulase, and blue pigment regulation. In plasmodium species, the PfCRT is situated at the intra-erythrocytic digestive vacuole. Mutations in this protein present verapamil-reversible chloroquine resistance to *P. falciparum*. The mutations in PfCRT result in increased compartment acidification. PfCRT-cognate vicissitudes in chloroquine replication involve altered drug flux across the parasite digestive vacuole membrane. Bray et al., concluded that PfCRT is mediated by the efflux of chloroquine from the digestive vacuole [72].

5. Analysis of single nucleotide variants scores

Three parameters have been considered for the estimation of structural changes of the PfCRT gene such as aggregation prediction (TANGO), amyloid prediction (WALTZ), and chaperone-binding prediction (LIMBO). SNPeffect 4.0: online prediction of molecular and structural effects of protein-coding variants was used for the study [83]. The three different positions of PfCRT gene were mutated manually in the protein sequence, that is, at 74, 75, and 76 positions. The mutated and the wild-type proteins were further processed using the SNPeffect software to calculate the difference in TANGO, WALTZ, and LIMBO score. The TANGO score obtained for the mutation of M74I, N75E, and K76T is given in **Figure 2**. The WALTZ scores of the wild type and the mutants are given in **Figure 3**. The LIMBO scores are given in **Figure 4**.

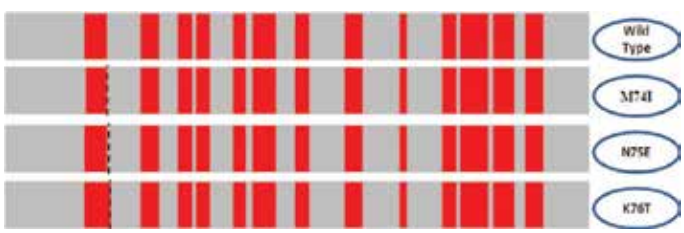

Figure 2. The aggregation prediction showing the differences of LIMBO scores of the wild-type PfCRT with the mutants.

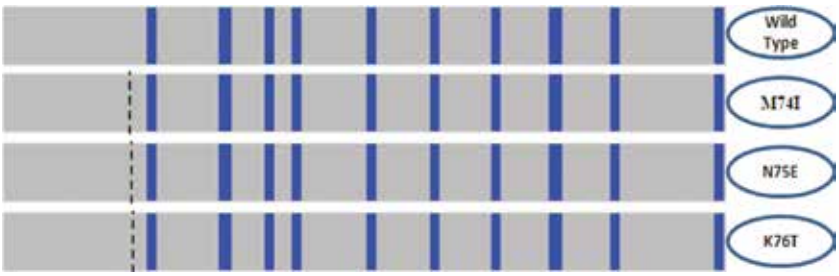

Figure 3. The amyloid prediction showing the differences of LIMBO scores of the wild-type PfCRT with the mutants.

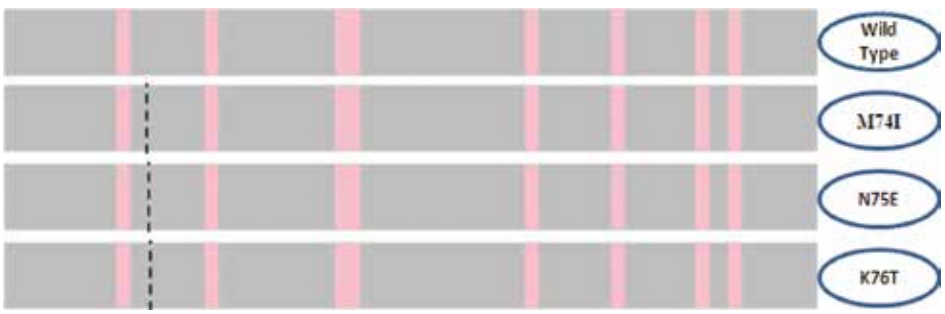

Figure 4. The chaperone-binding prediction showing the differences of LIMBO scores of the wild-type PfCRT with the mutants.

6. The multiple haplotypes of PfCRT

In South America and southeast Asia, the CQR *P. falciparum* was first came into sight in the late 1950s and early 1960s, leading to the suggestion of Su et al., that resistance rises from two independent basic events [61]. Further studies have been done to analyze a huge number of geographically diversed PfCRT alleles and microsatellite genotypes from parasite isolates have identified at least three additional independent foci of resistance [5, 6]. CQR creation has been discovered in the Thai-Cambodian border region still now (eventually spreading westward into Africa), Papua New Guinea, the Philippines, Colombia, and Peru [84].

Cooper et al. revealed that 21 exclusive CQR PfCRT protein sequences are identified from field isolates and two additional haplotypes have been created using CQ selective pressure on the 106/1 parasite line in laboratory [3]. It is impossible to differentiate the CQR foci or the genetic variations of the subsequent involved in one origin without understanding performing the whole analysis of the PfCRT sequence and its surrounding loci by means of microsatellite typing. Johnson et al. developed four unique CQS haplotypes using the drug selection procedures of a laboratory [85].

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Based on different geographical locations, the existence of the three PfCRT haplotypes revealed. According to Su et al. and Wootton et al. the first and the oldest resistant haplotype is CVIETIHSESII (amino acids 72–73–74–75–76–77–97–220–271–326–356–371), which exists in the FCB line of southeast Asia and is found in African isolates such as RB8, with consistency of spreading the CQR from Asia to Africa [5, 61]. The second haplotype is CVIETIHSESTI, which is found in the 102/1 Sudan strain, illustrated the characteristics of the isolates such as Dd2 from Thailand, and is newly explained the PH4 isolate from Morong, Philippines [2, 6]. An older PfCRT haplotype is CVIET found in South America, which implies that it may be because of the traveler who recently traveled to the location [86]. The third haplotype is detected in the INDO19, FCQ22, and 7G8 isolate line from Thailand, Papua New Guinea, and Brazil, respectively, and is reported as SVMNTIHSQDLR [2, 6, 7].

Different mutations in the PfCRT gene which change the nucleotide sequence into different genes and form different haplotypes are very general with the incidence of chloroquine resistant (CQR) [87].

7. Conclusion

The haplotype variations of PfCRT broadly classified into three groups, namely southeast Asian, Latin America, and Papua new guinea. This is used as a marker in the study of *P. falciparum* population diversity along with other markers. It is noteworthy to point out that PfCRT plays an important role in CQ transport and intracellular pH (pHi) regulation in parasite and the problem of drug resistance to antimalarials. In order to study the functions of wild-type PfCRT and mutants of PfCRT, calculation of different structural estimation score were generated where it showed that there is a notable variation in LIMBO, TANGO, and WALTZ scores.

Conflicts

The authors declared that there is no conflict of interest.

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Chapter 9

New Approaches for an Old Disease: Studies on Avian Malaria Parasites for the Twenty-First Century Challenges

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65347

Abstract

Emerging infectious diseases (EIDs) impose a burden on economies and public health. Because EIDs on wildlife are mainly affected by environmental and ecological factors, the study of EIDs in wildlife provides valuable insights to improve our understanding on their causes and their impact on global health. Malaria is an EID that has increased its prevalence in the last few decades at an alarming rate. Avian malaria parasites are abundant, widespread and diverse, which turn these parasites into an excellent model for the study of EIDs. In the face of new health and environmental challenges in the twenty- irst century, studies on avian malaria will provide new approaches for this old disease. The identfiication of essential genes for the malaria invasion, the study of modification of host behaviour by malaria parasites in order to promote the parasite transmission, and the knowledge of factors contributing to the emergence of infectious diseases in wildlife are essential for understanding parasite epidemiology, local patterns of virulence and evolution of host resistance. In this chapter, we will review the results of some recent investigations on these topics that will be useful for predicting and preventing EIDs in wildlife, livestock and humans.

Keywords: avian malaria, emerging infectious diseases, *Haemoproteus*, haemosporidians, *Plasmodium*

1. Introduction

Malaria is one of the world's deadliest diseases, with 214 million cases and an estimated 1 million malaria deaths every year. Although the first human recording of malaria was in China in 2700 B.C., most probably this disease is older than humans. Fossil evidence shows that modern malaria was transmitted by mosquitoes at least 20 million years ago, and the recent analysis of the pre-historic origin of malaria has suggested that earlier forms of the disease, carried by biting midges, are at least 100 million years old and probably much older [1]. Hence, malaria not only infects humans. In fact, systematic parasitologists have erected more than 500 described species belonging to 15 genera within the order Haemosporidia (phylum Apicomplexa) that infect reptiles, birds and mammals, and use at least seven families of dipteran vectors for transmission [2, 3]. These parasites are widely distributed in every terrestrial habitat on all the warm continents. Within these parasites, avian malaria is the largest group of haemosporidians by the number of species. They are widespread, abundant and diverse, and are easily sampled without disrupting the host populations. Although the term 'malaria parasites' has been a controversial issue among parasitologists, ecologists and evolutionary researchers [4, 5], authors usually include genera *Plasmodium*, *Haemoproteu*s and *Leuocytozoon* among the malaria parasites [4].

Investigations on avian malaria have contributed significantly to the knowledge on biology and ecology of malaria parasites of other vertebrates, including human malaria [6]. Since the discovery of the mosquito transmission of malaria in birds by Sir Ronald Ross, studies on malaria parasites of birds have saved millions of human lives. For example, essential advances in medical parasitology such as the development of anti-malarial compounds (e.g. plasmochin, primaquine and atebrin), the study of the life cycle and cultivation in vitro were initially developed using bird haemosporidian models.

Also, during the 2000s, research on bird malaria was at the very peak because scientists recognised the benefits of using studies on avian malaria to answer ecological, behavioural and evolutionary questions. Nowadays, far to be outdated, investigations on avian malaria will be essential to face new health and environmental challenges in the twenty-first century. In this chapter, we will review the newest contributions on bird studies helping in the fight against malaria.

2. Emerging infectious diseases and wildlife studies

In the last century, advances in vaccines and antibiotics, as well as other improvements in food intake and sanitation, contributed to the fast development in demography and economic growth in many parts of the world [7]. These advances brought the erroneous idea of a possible world without the burden of pathogens, followed by a flawed policy of reducing investment in the research of infectious diseases [8]. Pathogenic microorganisms rapidly evolve using multiple genetic evolutionary mechanisms, thus steadily adapting to new environments and escaping host's defences. As a consequence, more than 300 events of emerging and re-emerging

http://dx.doi.org/10.5772/65347

infectious diseases (EIDs) have killed millions of people since the 1940s and represent one of the major threats to human, livestock and wildlife in the twenty-first century [9]. These diseases are caused by pathogens from animals that now infect humans (HIV-1), or pathogens that have been probably presented in humans for centuries, but continue to appear in new locations (Lyme disease) or have evolved resistance to drugs (malaria resistance to chloroquine, mefloquine and artemisinin), or that reappear after apparent control or elimination (tuberculosis). Ironically, the health improvements and economic developments of the last century also contributed to the increase of these pathogenic diseases, as 'hidden costs' of this wellness. The economic and demographic growth led to millions of people live in crowded urban areas, thereby facilitating the spread of infections [10]. Also, the deforestation for logging and farming in tropical rainforests to meet the demands of growing population have provoked changes in the ecology and epidemiology of vector-borne diseases (e.g. malaria, leishmania and Chagas disease), thus favouring the spread of the disease [11].

Studies on wildlife may provide essential information in the fight against EIDs for several reasons. On the one hand, wildlife is an essential component in the epidemiology of many EIDs. In this sense, more than 60% of these diseases in humans are caused by pathogens spread from animals, and 71.8% of these zoonotic diseases events are provoked by pathogens with a wildlife origin [9]. On the other hand, socio-cultural and economic drivers (e.g. population density, economic growth), as well as ecological and environmental conditions (wildlife species richness, rainfall), may be major determinants of surge and spread of diseases in humans. In opposition to human studies on EIDs, wildlife studies are free of socio-economics and cultural confounding variables, thus providing reliable conclusions on the ecological drivers of the epidemiology.

2.1. Avian malaria and deforestation

Infections with vector-borne pathogens have become one of the main EIDs in the last years. Arthropods such as mosquitoes, ticks and bugs are responsible for transmission of viruses (dengue, chikungunya, Zika), bacteria (Lyme disease) and protozoans (malaria, Chagas). Anthropogenic deforestation and land use change have been proposed to cause the spread of vectors and the re-emergence of malaria in South America [12]. In this sense, it has been shown that the biting rate of *Anopheles darlingi* (primary human malaria vector in the Amazon) in deforested rainforest sites was more than 278 times higher than the rate determined for areas that were predominantly forested [13]. Also, it has been reported that deforestation can increase human malaria prevalence up to 50% [14]. However, as mentioned previously, the drivers favouring malaria outbreaks go beyond the basic biological elements and include ecological as well as socio-economic factors [15]. Because these puzzling effects are irrelevant in the context of wildlife malaria parasites, further studies (e.g. avian malaria studies) removing potential confounding variables are required to confirm whether anthropogenic land-use change is a key driver of disease emergence.

In recent years, several studies have analysed the effects of habitat fragmentation and deforestation on the prevalence of bird haemosporidian parasites in different continents. Bonneaud et al. [16] examined the prevalence of infection of bird malaria in both pristine and

disturbed forests from Cameroon, showing a higher prevalence of *Plasmodium* lineages in pristine as compared with disturbed forest sites. Also, Chasar et al. [17] analysed the diversity, prevalence and distribution of avian haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) from nine-paired sites (disturbed vs. undisturbed habitats) in Southern Cameroon in two widespread species of African rainforest birds. They found that the prevalence of *Haemoproteus* and *Leucocytozoon* infections was significantly higher in undisturbed than in deforested habitats. They also showed that the prevalence of *Plasmodium megaglobularis* was higher in undisturbed areas, whereas the prevalence of infection of *P. lucens* was higher in deforested areas. Furthermore, Loiseau et al. [18] reported the variation of parasitaemia intensity and co-infections of avian haemosporidian parasites in two common African bird species at three sites with distinct habitat characteristics in Ghana. They detected a variation in infection prevalence and intensity of parasitaemia that differ in environmental factors; thus suggesting that spatial heterogeneity can impact the prevalence, frequency of co-infections, and chronic parasitaemia intensity of haemosporidian parasites.

In Hawaiian Islands, malaria is thought to be responsible of the population decline and even extinctions of many native bird species [19]. In addition, deforestation could also have contributed to the population decimation by altering the patterns of malaria transmission [20, 21]. In this sense, it has been suggested that deforestation of the Alaka'i Wilderness Preserve on Kaua'i Island could have changed the pattern of seasonal transmission of avian malaria to a pattern of continuous transmission through all the year [20], which could enormously increase the prevalence and pathogenic effects of avian malaria.

Moreover, Laurance et al. [22] investigated the effects of habitat fragmentation and ecological parameters on the prevalence of malaria parasites (genera *Plasmodium* and *Haemoproteus*) in bird communities of Australia. They analysed the prevalence and genetic diversity of haemosporidians across six study sites including large sites and continuous-forest sites, finding that the prevalence of the dominant haemosporidian infection (*Haemoproteus*) was significantly higher in continuous forest than in habitat fragments.

In Brazil, Belo et al. [23] examined the presence and genetic diversity of haemosporidian parasites in 676 wild birds from three different environmental regions (intact cerrado, disturbed cerrado and transition area Amazonian rainforest-cerrado) with the aim to determine whether different habitats are associated with differences in the prevalence and diversity of malaria infection. Surprisingly, they found that neither the prevalence nor the diversity of infection of *Plasmodium* spp. or *Haemoproteus* spp. differed significantly among the three habitats studied. More recently, Ricopa and Villa-Galarce [24] have studied the prevalence and genetic characterisation of avian malaria lineages in one disturbed and one preserved area of National Reserve Allpahuayo-Mishana in Peruvian Amazon. They found a higher prevalence and a higher diversity of malaria infection in birds from the deforested area when compared to birds from pristine forest.

2.2. Avian malaria parasites as emerging infectious diseases: the role in biological invasions

Alien, also called non-indigenous, exotic or non-native species, are defined as those species that colonise an area beyond their natural range, where they reproduce and establish a

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population. In addition to urbanisation, demographic growth and land-use change, the introduction of domestic and wildlife alien species can also provoke emerging diseases with tremendous costs in terms of loss of biodiversity, mortality and economic expenses [25]. In this sense, several studies have shown zoonosis linked to birds spreading diseases to humans. For example, it is thought that the West Nile virus, a bird pathogen but also causing mortality to humans, was introduced to New York by migratory or invasive bird species [26]. Also, the bird flu virus (H5N1), which has been registered, was transported by invasive bird species [27]. But despite these negative impacts of invasive species and the efforts from scientists to understand biological invasions, the mechanisms that allow one species to become invasive are still poorly understood.

Since the eighteenth century, more than 1400 human attempts to introduce 400 bird species have been recorded worldwide [28]. But not all of these introduced birds have resulted in established populations of invasive birds in the new regions. In fact, only 10% of introduced species are able to colonise new environments and become successful invaders [29]. In consequence, some life history traits and ecological attributes could allow some alien species to maintain high survival and reproductive success in new locations and to become successful invaders [28]. It has been proposed that parasites may play this role facilitating the successful colonisation of their bird hosts [30]. Hence, parasitic infections (or sometimes, their absence) may facilitate or limit invasions impacting native species via both direct and indirect effects. Three hypotheses (novel weapon hypothesis, enemy release hypothesis and biotic resistance hypothesis) have been proposed to explain the role of parasites in invasions in bird- parasite systems (**Table 1**). On the one hand, exotic bird species can act as a 'Trojan horse' because they can bring alien parasites and pathogens inside them, which could favour the dissemination in the new areas of their avian host species. In the history, pathogens have played a role bringing diseases in humans that become epidemic in susceptible native populations [39]. As example, in European conquest in the Americas smallpox spread rapidly killing an estimated 95% of the indigenous population far in advance of the European themselves [39]. Following this idea, the novel weapon hypotheses (NWH) states that invasive species gain advantages over native species by bringing their own parasites to the new environments against which the introduced species but not the natives have evolved defences [40, 41]. These co-introduced parasites may switch to native bird hosts and spread in the new communities, hence becoming themselves invasive parasites provoking serious damages to indigenous bird species. But the role of parasites in invasions may extend well beyond such direct effects, and hence some indirect effects may also be expected. In this sense, the enemy release hypothesis (ERH) states that non-native bird species could become invaders because they have lost their co-evolved malaria parasites during the process of colonisation, and thus they may increase their competitive ability and displace native species in the new areas. Conversely, the biotic resistance hypothesis states that native parasites in the indigenous species may reduce the fitness of the potential bird invader and prevent its spread and establishment. Next, we will focus on the role of avian malaria parasites co-introduced with their bird hosts.

Avian malaria parasites are among the most pathogenic species of poultry and wildlife birds, being responsible for economic losses, mass mortality, population declines and even extinc-

tions of many bird species worldwide after its introduction outside its native range [42]. For all these reasons, the International Union for Conservation of Nature (IUCN) classifies avian malaria to be among the 100 of the world's worst invasive alien species [43]. The spread of exotic avian malaria in Hawaiian Islands is the best documented example of the effects of invasive malaria on native bird communities. Since the discovery of Hawaiian Archipelago by the Captain Cook in 1774, more than a half of over 100 endemic bird taxa in Hawaii have been driven to extinction by a combination of habitat loss, introduced species and diseases [19, 44]. In 1826, the primary vector for avian malaria *Culex quinquesfasciatus* was accidentally introduced in Hawaii from shipping vessel HMS Wellington [19, 45]. The colonisation of this mosquito species, as well as the introduction of non-native bird species co-transporting avian malaria *Plasmodium relictum*, then provoked a wave of extinctions and endangerment among Hawaiian forest birds since the 1920s [21].

Novel weapon hypothesis (NWH)	**Reference**	**Observations**
Hawaii	van Ripper et al. [21]	
Hawaii	Atkinson et al. (2005) [46]	
Hawaii	Lapointe (2005) [47]	
Hawaii	Atkinson and Samuel (2010) [48]	
New Zealand	Doré (1920) [49]	
New Zealand	Tompkins and Gleeson [31]	
New Zealand	Barraclough et al. [32]	
New Zealand	Howe et al. [33]	
New Zealand	Ewen et al. (2012) [50]	
New Zealand	Schoener et al. (2014) [51]	
Galapagos Islands	Levin et al. [34]	
Galapagos Islands	Santiago-Alarcón et al. [35]	
Galapagos Islands	Levin et al. [36]	
Perú	Marzal et al. (2015) [52]	
Enemy release hypothesis (ERH)		
Southern Asia	Beadell et al. (2006) [53]	Mixed results with NWH
Seychelles Islands	Hutchings (2009) [54]	
Brazil	Lima et al. (2010) [55]	
6 continents (58 locations)	Marzal et al. [37]	
Biotic resistance hypothesis		
Lesser Antilles	Ricklefs et al. (2010) [56]	
sGarcía-Longoria et al. [38].		

Table 1. Main studies on the role of avian malaria parasites in the global spread of their bird hosts.

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Similar to Hawaii, recent investigations have detected avian malaria *Plasmodium* spp. in New Zealand birds, thus suggesting that avian malaria could be an emerging threat to New Zealand avifauna [32, 33]. For example, an outbreak of malaria has killed more than 90% of the population of the endemic Yellowheads (*Mohoua ochrocephala*) [46]. Also, Howe et al. [33] have reported the death of native and exotic bird species due to acute *Plasmodium* spp. infection. In addition, Baillie et al. [46] have documented three exotic *Plasmodium* species infecting the endemic New Zealand passerine Bellbird *Anthornis melanura*. Finally, it is known that four alien species of mosquitoes have been established and rapidly spread in New Zealand [31, 47] and are likely to be the vectors responsible for some avian malaria outbreaks in the New Zealand [48].

Malaria parasites were historically considered to be absent in Galápagos Islands, because studies based on microscopic and molecular screening of parasites failed to detect malaria parasites in Galápagos birds [49, 50], most probably due to the absence of competent vectors. However, in the last decade, several studies have showed malaria-infected birds in several islands in the archipelago, thus suggesting recent arrival of avian malaria parasites. In this sense, the only known competent vector for *Plasmodium* parasites present in the archipelago is the mosquito *Culex quinquesfasciatus*, which was described for the first time in Galápagos in 1989 and it was well established by 2003 [51]. Following the mosquito introduction, the first report of malaria-infected birds in the Galápagos Archipelago come from Levin et al. [34], identifying penguins as positives for *Plasmodium*. Later, Santiago-Alarcon et al. [35] showed haemosporidian parasites infecting the endemic Galápagos dove (*Zenaida galapagoensis*). More recently, Levin et al. [35] have found different genetic lineages of *Plasmodium* parasites infecting Galápagos birds. Some of these lineages seem to be transient infections of parasites not established on the archipelago, whereas other parasite lineages are thought to be established and regularly transmitted in the archipelago.

P. relictum is an avian malaria parasite with highly virulence, genetic diversity and markedly invasive nature [19, 45, 52]. Recent molecular studies on partial sequences of the cytochrome oxidase b gene on this parasite have revealed different genetic diversity of this parasite, with two main parasite lineages: *P. relictum* GRW4 and *P. relictum* SGS1. *P. relictum* GRW4 is the parasite lineage responsible for devastating epizooties reported in Hawaii and New Zealand, and has a broad geographical range including Africa, Asia and the Americas [37, 45]. Its sister lineage, *P. relictum* SGS1 is widespread and actively transmitted in Europe, Africa and Asia [37], but until very recently, this invasive lineage had not been reported in the mainland Americas [37, 45, 53–55].

Marzal et al. [56] have recently showed the first report of this invasive pathogen in the mainland Americas. They analysed more than 100 blood samples from native bird species from South America, showing the presence of *P. relictum* SGS1 in neotropical birds from two different areas of Peru. In this study, *P. relictum* SGS1 was also geographically spread, and the most generalist and prevalent parasite lineage found in the study, infecting 13 individuals from eight host species (39.40% of the total infections). We must be particularly aware on the presence of this invasive parasite in birds of South America because it may represent a serious risk to this avifauna and a potential threat to over one-third of all bird species in the world.

Would it be possible to eliminate emerging infectious diseases? We do not think so. Indeed, it seems unlikely that most emerging infectious diseases will be eradicated in a close future [57]. In the fight against emerging infectious diseases, we have to follow the advice of the Red Queen to Lewis Carroll's Alice in Wonderland: '…it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!' Therefore, this is a continuous process, where we have to keep on researching to avoid being out of step in the fight against malaria and other emerging infectious diseases. Studies on avian malaria research may play an essential role in these investigations to determine the key factors (e.g. deforestation and land-use change, biological invasions) contributing to the emergence of these diseases.

3. Identification of malaria genes

Approximately, 40% of the world population lives in areas where malaria is transmitted. In some areas, as sub-Saharan Africa, malaria may cause a rate of mortality in 5-year-old children around 90% [58]. An important tool in the fight against malaria parasites is the identification and sequencing of malaria genes that could give essential information about this harmful disease. Hence, in 1996, an international effort was launched to sequence the *Plasmodium falciparum* genome with the aim to open new paths for researching and for the development of new treatments and vaccines against malaria parasites. Some years later, the results were published showing that there was possible to sequence all the chromosomes of *P. falciparum* clone 3D7 [59–61]. The results showed that these chromosomes encode for 5300 genes and are the most (A + T)-rich genome sequence to date.

Moreover, these chromosomes possess a high number of genes related to immune evasion and host-parasite interactions and fewer enzymes and transporters. In the short term, however, it has been suggested that the genome sequence alone provides little information about malaria parasites. As Gardner et al. [59] claim 'much remains to be done'. These results might need to be accompanied by new methods of control, including new drugs and vaccines, improved diagnostics and effective vector control techniques. In this section, we will deal with some malaria genes that are essential for identification of malaria transmission areas and to develop strategies to avoid spread of the disease.

Also, we will show some recent studies on the identification of malaria genes that are crucial in the parasite life cycle, and could be used as a target for future vaccines or anti-malaria drugs.

3.1. Identification of merozoite surface protein gene 1 (MSP1) and malaria transmission areas

Malaria parasites have an important effect in populations where it is present affecting the survival of the community and la*y*ing out with facility [5]. Therefore, there is a general need to define populations at risk for appropriate resource allocation and to provide a robust framework for evaluation its global economic impact. The interest of mapping the global distribution of malaria parasite has increased in the last decade. Some authors have decided

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to identify some essential genes with an enormous variability in order to distinguish different location of the malaria parasites [62]. A commonly used good candidate gene is merozoite surface protein 1 (msp1). This gene is one of the most variable genes found in the human malaria parasite *P. falciparum* [63]. It encodes a protein involved in the attachment of the malaria parasite to the host red blood cell [64]. Within *P. falciparum,* the msp1 gene encodes for a 190 kDa protein that is separated in four different polypeptides (p83, p42, p38 and p30). During the erythrocyte invasion, the polypeptide p42 is split in two more polypeptides (p19 and p33). However, at the end of the process the only polypeptide that remains into the erythrocyte is p19 [65]. This gene has been analysed in a wide number of studies with the aim to determine whether it is a good candidate for developing vaccines or anti-malaria drugs. In this sense, [66] showed that antibodies to the p19 peptide are found in populations with high malaria prevalence and can be associated with immunity to the malaria parasite.

In 2013, Hellgren et al. [67] identified this gene in *P. relictum* SGS1, GRW11 and GRW4 lineages. They found that, within msp1 gene, there are nine different alleles split into the three

P. relictum lineages: SGS1 (alleles Pr1, Pr2, Pr3 and Pr7), GRW4 (alleles Pr4, Pr5, Pr6, Pr8 and Pr9) and GRW11 (alleles Pr2, Pr3 and Pr7). Theses alleles have a specific distribution around the world affecting areas as South America, Europe, Asia or Africa [68]. Moreover, they suggested that due to its high variability, this gene could be used as a candidate to investigate how different host species cope with the infection. In this sense, Hellgren et al. [68] confirmed some transmission events by the lineages analysing resident bird species or juvenile individuals before migration. Therefore, some alleles are restricted to a specific area. For instance, the allele Pr1 is, to date, only present in sub-Sahara areas, while the Pr2 allele has been mainly detected in European areas. This strict allocation may suggest the existence of transmission barriers (e.g. vector communities or abiotic factors) limiting transmission between regions.

By barcoding the msp1 gene in SGS1 and GRW4, a recent study has determined whether haemosporidian transmission in house martins occurs at European sites by sampling juvenile birds house martins (a migratory species with a high fidelity to its area of hatching and nesting) [30].

Moreover, they analysed the msp1 alleles in both adult and juvenile house martins in order to identify their potential areas of transmission. Surprisingly, their results showed that some juvenile and adult house martins were infected by Pr2 allele of *P. relictum* SGS1, an allele thought to be exclusively transmitted in Europe. These results showed, for the first time, that juvenile house martins may become infected with *Plasmodium* parasites already before their first migration to Africa, thus confirming that active transmission of *Plasmodium* spp. to house martins also occur in Europe. These findings emphasise not only the importance of using multiple independent loci of avian *Plasmodium* parasites to understand transmission areas of blood parasites but also the use of birds as study model in parasite analyses.

3.2. New target to avoid completion of malaria life cycle: the chitinase gene (CHT1)

Malaria parasites (including human malaria) show a complex life cycle that requires mechanisms adapted to enable the parasite invasion into the different tissues from the vertebrate host

to the arthropod vector. Apparently, arthropods develop a protective peritrophic membrane (PM) against pathogens around their midgut after each blood meal. This PM blocks the penetration of blood parasites and avoids the spreading of the parasite to other organs. In turn, malaria parasites have developed a mechanism to overcome the PM barrier. Once the malaria parasite has complete its sexual stage in the mosquito stomach, the ookinete has the ability to cross the PM by secreting a chitinase that has catalytic and substrate-binding sites breaking down this layer. After crossing the PM, ookinetes finally transform into oocytes, which after maturing releases the sporozoites that move to the salivary glands where they are ready for infecting a new host [69]. Therefore, the role of the chitinase gene is essential in the life cycle of malaria parasites.

The chitinase gene has been study for years due to the variability of structures that it shows. In mammals, some *Plasmodium* parasites may present two different structures. For example, in human malaria (*Plasmodium vivax*), shared human and primate malaria (*Plasmodium knowlesi*) and in rodent malaria (*Plasmodium berghei*, *Plasmodium yoelii* and *Plasmodium chabaudi*) the chitinase gene presents a long structure and has a catalytic domain and a chitin-binding domain. However, the chitinase gene in human *P. falciparum* and in primate *Plasmodium reichenowi* presents a short structure and lacks the chitin-binding domain [70]. Concerning birds, it has been shown that *Plasmodium gallinaceum* has functional copies of both the long (PgCHT1) and the short (PgCHT2) chitinase gene suggesting that *P. gallinaceum* could be a common ancestor of the mammalian *Plasmodium* parasites that subsequently lost either the short or the long copy of this gene. However, the phylogenetic relationship among *Plasmodium* parasites infecting mammals and birds has been intensively debated over the past decades. On the one hand, some authors suggest that *P. falciparum* is closer phylogenetically to bird parasites than other mammalian malaria parasites [71]. On the other hand, several authors claim that mammalian parasites form a monophyletic clade [72]. Additionally, it has been suggested that *P. gallinaceum* is not the most prevalence parasite in birds and new studies would be needed in order to clarify this relationship. In this sense, Garcia-Longoria et al. [73] identified the chitinase gene in one of the most harmfulness and widespread avian malaria parasites, *P. relictum*. They demonstrated that *P. relictum* presents both copies encoding for chitinase (PrCHT1 and PrCHT2), thus supporting the hypothesis that avian malaria parasites could be the ancestor for the chitinase gene in malaria parasites of primates and rodents. Therefore, given the current phylogenetic hypothesis, it could be assumed that mammalian parasites evolved from an avian parasite that carried two copies of the chitinase gene. These findings are quite remarkable if we take into account that the evolutionary pathway of the malaria parasites is being decoded day-by-day, and the ancestors of human malaria parasites may give essential gene information that could be used for development of new anti-malaria treatment.

4. Malaria parasites and escape behaviour

Behavioural traits are an important factor in the life cycle of all live organisms. Behaviour may determine whether an individual ends up as a survivor or as a prey [74] or it can even acts as a defence against some parasites. For example, some organisms modified their behaviour by

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plastically changing their life history in order to evade parasite or to minimise the impact of infection [75]. A good example of behaviour as a defence mechanism to avoid is displayed in *Drosophila melanogaster*. Since small larvae are better protected from parasites, this fly can reduce the size of their larvae in habitats with high concentration of pathogens [76]. Ants also can modify their behaviour as a defence against parasites, as they reallocate their nests more often in areas where parasites are common [77]. All these examples suggest that behaviour is an essential key in the survival of individuals.

Anti-predator behaviour is a specific kind of behaviour that is consistent in the presence of a predator across time and contexts [78] and imposes an important selection pressure on preys [79]. Thus, when a predator stalks a prey, the first mean of avoidance of predation is escaping from the predator. However, when the prey is already captured by the predator, the behaviour displayed by the prey can be much more specific: the escape behaviour. Among others, escape behaviour includes several behavioural traits such as (i) the intensity with which a captured individual wriggles to escape, (ii) biting or attacking the predator,(iii) whether it loses feathers, limbs, or a tail and thereby manages to escape, (iv) fear screaming and (v) akin behaviour to feigning death [80]. These variables are closely related to the probabilities that one individual has to escape; thus, the more intense this escape behaviour in one individual is, the more probabilities would have this individual to escape from the predator. For example, a prey individual may emit a loud fear scream that can either warn co-specifics or to attract secondary predators, thus facilitating the escape [81]. Additionally, in 2011, Møller et al. [82] showed that birds with high levels of predation wriggled more when captured by a human, hence increasing their probabilities to escape from the predator, showing that intense escape behaviour is related with high levels of predation.

In addition to predation, parasite represents another major cause of mortality in birds. Some studies demonstrated that the presence of parasites and predators may provoke stress to animals and reduce the immune function in one individual suggesting a relationship between malaria parasites and predation [83]. Later, Møller and Nielsen [81] showed that individuals infected with blood parasites showed lower intense of escape behaviour. Both studies reveal an underlying mechanism that links predation to prevalence of blood parasites. Recently, this underlying mechanism has been analysing. In this sense, Garcia-Longoria et al. [84] tested whether species with higher prevalence of *Haemoproteu*s, *Plasmodium* or *Leucocytozoon* infection differed in escape behaviour from species with lower prevalence. They found that some escape behaviours are positively related with the prevalence of these blood parasites, where bird species with an intense escape behaviour showed higher parasitaemia of blood parasites. The observed correlation between blood parasite infections and escape behaviour may suggest a correlation between host behaviour and parasite infection, where individuals showing brave behaviours (e.g. exploratory behaviours or escape behaviours) could increase their likelihood of become infected by increasing the chances of co-specific encounters, injuries or vector bites. Alternatively, this correlation may also show a possible manipulation by the parasite of the behaviour of its host to enhance its chances of transmission.

Regarding to this latter hypothesis, the behavioural manipulation hypothesis posits that manipulation of host behaviour by parasites may confer fitness benefits to the parasite, usually

increasing transmission success to the parasite [85]. There are some studies supporting this hypothesis. One of the most well-known examples is the manipulation displayed by *Toxoplasma gondii*, a blood parasite that may infect rodent and other mammals. A mice infected with *T. gondii* lose its fear against feline predators (the definitive host of *T. gondii*), thus increasing the likelihoods of transmission of the parasite to its final host [86]. Concerning malaria parasites, little is known about whether malaria parasites can manipulate the behaviour of their avian host. Malaria parasites show a complex life cycle with both vertebrate (e.g. birds, reptiles and mammals) and invertebrate host (e.g. mosquitoes). In 2013, Cornet et al. [87] demonstrated that infected birds attract more vectors than uninfected ones suggesting that malaria parasites may modify the behaviour of their vector in order to increase their own transmission. However, whether malaria parasites may manipulate escape behaviour of their vertebrate host has been poorly analysed. To date, only one study has explored the association between avian malaria parasites and behaviour in the vertebrate host [88]. Garcia-Longoria et al. [88] experimentally tested whether malaria parasites could manipulate the escape behaviour of their bird hosts. They experimentally infected house sparrows with *P. relictum* and measured the escape behaviour of sparrows before and after the malaria infection. They showed that experimentally infected individuals increased the intensity of their escape behaviour after the parasite inoculation, hence demonstrating that *P. relictum* may modify the escape behaviour of their avian hosts. These results agree with the behavioural manipulation hypothesis, because the facility to escape from predators would indirectly increase the transmission of the parasite, as well as enhance the survival of the hosts. One remaining question concerns the identification of the mechanisms that malaria parasites may use to boost the escape behaviour of their avian hosts. In this sense, some parasites are known to modify the behaviour of their host by secreting substances capable of altering the neuronal activity of the host. For example, the trematode *Schistosoma mansoni* secretes opioid peptides that change the function in the brain of its hosts by producing cystic fibrosis causing the necrosis of some brain areas. Other parasites, like helminths, can alter the concentration of serotonin or dopamine in the host, thus altering some neurological mechanism in their host. Hence, the mechanisms that *P. relictum* might use in order to modify the escape behaviour of its avian host it remains unknown. Future studies on avian malaria research should go deeper with the aim to identifying the mechanisms underlying the behaviour alterations of their bird hosts after malaria infection.

5. Conclusions

Malaria parasites and other emerging infectious diseases are one of the major challenges for global health in the twenty-first century. Despite the efforts made by scientist and health care providers, malaria parasites are becoming drug-resistant, as well as they are boosting their mortality rate in some regions and increasing their areas of transmission. These risings can be provoked by some anthropogenic alterations as deforestation or biological invasions, thus provoking changes in the ecology and epidemiology of vector-borne diseases and outbreaks in human, livestock and wildlife emerging infectious diseases. Moreover, in human malaria studies, it is very difficult to assess if the changes in parasite prevalence are due to socio-

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ecological factors or to the effects of environmental alterations. These facts emphasise the importance of the study of malaria parasites in wild animals, free from social and economic factors, to fight against this pathogen. Here, we have also focused our attention in the identification of new avian malaria genes that could help in the detection of malaria transmission areas around the world. In addition, the identification of malaria genes with high genetic variability will supply essential information in the evolution of both human and avian malaria pathogens and would provide scientists with new tools for the development of anti-malaria drugs. Our current knowledge about malaria and EIDs is still limited. Further investigation and exploration are needed in order to gain a better understanding of the malaria distribution and the global economic and health impact of malaria. Moreover, it is important to increase awareness of the consequences of introducing non-native species in different habitats and to increase the control in biosecurity borders for avoiding the introduction of alien pathogens such as malaria parasites. Finally, we should be fully aware that there is no ending in the fight against EIDs, where 'it will take all the running (*researching*) you can do, to keep in the same place'.

Acknowledgements

This study was funded by research projects of the Spanish Ministry of Economy and Competitiveness (CGL2015-64650P) and Junta de Extremadura (GRU15117). Sergio Magallanes was supported by a PhD grant from Ministry of Economy and Competition of Spain.

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Section 3

Therapeutics and Antimalarials

Chapter 10

Approaches, Challenges and Prospects of Antimalarial Drug Discovery from Plant Sources

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65658

Abstract

Nearly 3.3 billion people globally are at risk of malaria, with 1.2 billion being at high risk. Children under 5 years of age and pregnant women in sub-Saharan Africa still account for a higher percentage of malaria-related mortalities, despite recent reports of decline in malaria mortalities in Africa. Majority of these deaths are caused by *Plasmodium falciparum*, a lethal malaria parasite which has developed resistance to different classes of antimalarial drugs and is responsible for complicated, severe disease. To forestall the debilitating impact of the disease and provide safe and effective alternative therapies, medicinal plants have been explored as a source of new antimalarials. The isolation of quinine and artemisinin from plants present medicinal plants as a robust source of effective antimalarials. In this chapter, we review the different approaches employed in antimalarial discovery from plants, different classes of plant antimalarial compounds and their proposed mechanisms of action. Compounds that show potential for further development based on their high efficacy and selectivity are also highlighted. Common obstacles encountered in the process of antimalarial drug discovery from plant sources are identified and prospects for the identification of new, effective antimalarial components from plant sources are also discussed.

Keywords: Antiplasmodial screening, Antimalarial, Medicinal plants, *Plasmodium falciparum*, Selectivity

1. Introduction

Malaria has remained a leading cause of mortality in close to 100 countries where nearly 2.4 billion people reside, almost half of whom are located in sub-Saharan Africa. With continuous malaria transmission all year round and increasing rates of human movement

in disease-endemic areas, a high burden of antimalarial use in these areas has contributed to global malaria burden [1]. Exposure of parasites to suboptimal antimalarial drug concentrations favors the selection of parasites with traits that enable them to survive in the presence of the drug [2]. Over time, the most lethal strain of the malaria parasite, *Plasmodium falciparum*, has developed resistance to many classes of antimalarial drugs and this contributes to the development of severe malaria complications which can be fatal without prompt treatment. Other less lethal strains, *P. ovale* and *P. vivax*, can exist as latent hypnozoites in the liver which can initiate a relapse months to years after the initial infection [3]. With increased global warming, it has been predicted that the geographic area covered by the malaria vector, *Anopheles* will increase species and this will cause wider transmission of malaria parasites that are resistant to most of the antimalarials presently available [4]. Thus, it has become necessary to develop versatile, robust alternatives to current antimalarial regimens that would be effective against all malaria parasite strains. Within the last decade, efforts have been made to identify new strategies to prevent and treat malaria [5]. Plants have been identified as a robust source for antimalarial drug discovery and interestingly, cinchona alkaloids (such as quinine and quinidine) and artemisinin obtained from plants are still clinically relevant today for the treatment of severe malaria [6].

In order to fast-track the development of effective, alternative medicines from medicinal plants, appropriate pre-clinical studies that confirm their safety and efficacy are required to provide sound experimental data that establish an evidence base. The development of effective medicines from plants is not without its challenges and efforts should be made to address these especially with novel approaches to preclinical screening and clinical testing. Conventional drug development is very time dependent and cost dependent but is rarely rewarding eventually; as the number of approvals for new drugs has declined in recent years [7]. Hence, we also explore the revisited "reverse pharmacology" paradigm to address this problem and secure the future of antimalarial drug discovery.

1.1. Current status of drug discovery from plant sources

Many medicines used against different diseases including cancer, diabetes, hypertension, neurodegenerative disorders and infectious diseases have been sourced from a plant or designed based on scaffolds of compounds isolated from plants. The latest of these include artemether (antimalarial), galantamine (for Alzheimer's disease), nitisinone (for tyrosine-associated metabolic disorder) and tiotropium (anticholinergic), which have all recently been introduced in the United States or are currently involved in late-phase clinical trials [8]. Drug discovery from medicinal plants involves a multi-thronged approach that includes, but is not limited to traditional medicine practitioners, botanists, medicinal chemists, pharmacologists and molecular biologists. Conventionally, plants are selected either randomly or based on their claimed historical medicinal relevance and subjected to sequential extraction and purification steps. This can be very tedious and time-consuming and more effective methods for identifying new lead molecules from plants have been explored. These include chemoinformatics and bioinformatics as tools for in silico drug discovery [9], systems/polypharmacology approach which integrates oral bioavailability tests, druggability, blood-brain barrier permeation, target

http://dx.doi.org/10.5772/65658

identification and network analysis owing to the complex composition of medicinal plant extracts and their diverse physiological effects [10]. High throughput pharmacological screens and genetic manipulation have also been applied to discover new drug leads from plants, in which plants extracts are screened against an array of receptors with or without gene manipulation and compared to existing drugs [11].

2. Approaches in antimalarial drug discovery

Six major approaches to antimalarial drug discovery have been identified and reviewed, including the investigation of natural products [12]. A plant-based approach is particularly useful in resource-poor, malaria-endemic areas where nearly one-fifth of patients rely on herbal remedies to treat malaria and febrile illnesses [13]. The choice of plants for antimalarial drug discovery may be based on both random and empirical methods to explore biodiversity or through studies guided by traditional use of the plant in the treatment of fever. The latter ethnopharmacological approach has been recognized to give higher success rates for finding active compounds, as over 50% of extracts from ethnomedicinal plants were active in vivo and/or in vitro [14].

2.1. Ethnopharmacology-based plant selection and extraction

Herbal medicines have played a pivotal role in health and disease management for many centuries. Different ancient civilizations, including Mesopotamian, Indian ayurveda, ancient traditional Chinese medicine and Greek unani medicine, show documented evidence for the use of herbs in the treatment of different ailments. In Africa, knowledge of traditional medicine constitutes part of a wholistic system, passed through generations by oral communication and indigenous practices [15]. The scientific exploitation of herbs used ethnomedicinally for pain relief, wound healing and abolishing fevers has resulted in the identification of a wide range of compounds that have been developed as new therapeutics [16].

The major role of ethnopharmacology is to discover new plant-derived compounds based on the traditional use of medicinal plants. The knowledge on the use of plants for fevers and other symptoms of malaria is used to guide the selection of plants to be subjected to antimalarial screening and isolation of active constituents. This is a favored and conservative approach in drug discovery as historical use of a plant as medicine increases the possibility that safe and pharmacologically active compounds would be isolated from it.

2.2. Preclinical efficacy studies

2.2.1. In vitro assays

In vitro cultures of asexual forms of *P. falciparum* are generally maintained in leukocyte-free erythrocytes at 2–5% hematocrit, in Roswell Park Memorial Institute (RPMI) culture medium supplemented with 5–10% human serum at 37°C under reduced oxygen conditions [17]. Advantages associated with this assay are the small amount of test sample required and its

flexibility, as it has been adapted for high — throughput screening of large compound libraries. With latest developments in image processing and automation technology, screening against live parasites in host cells can also be run in 1536-well formats. Also, concentrations producing 50% inhibition (IC_{50}) and 90% inhibition (IC_{90}) can easily be obtained from drug-response curves by nonlinear regression. Drawbacks to these assays include the need for continuous parasite culture and more importantly, the exclusion of host in vivo factors which affect drug disposition and action.

Detection of parasite growth in in vitro assays generally involves the examination of Giemsa-stained smears for viable parasites. This method is very time-consuming, lacks precision and limits rapid, large-scale screening of compounds. Colorimetric determination of parasite lactate dehydrogenase in the presence of nitro blue tetrazolium which is reduced to a formazan derivative has been developed and used successfully [18]. Other methods have been developed which rely on incorporation of radiolabeled metabolic precursors, measurement of dye-stained parasite DNA by fluorimetry or flow cytometry and use of luminometry for genetically modified parasites that express luciferase [19–21]. Fluorescence-based assays that employ DNA-binding fluorophores have also been described, for example, the fluorimetric method described by Benoît et al. [20] in which parasite growth is quantified by stained DNA of viable parasites. Enzyme-linked immunosorbent assays (ELISAs) with monoclonal antibodies which measure *P. falciparum*–specific antigen histidine-rich protein 2 (HRP2) or *Plasmodium* lactate dehydrogenase (pLDH) protein as index of parasite growth have also been reported [22].

Culture conditions for other human and nonhuman *Plasmodium* species are reviewed in detail elsewhere [23]. The culturing of exoerythrocytic sporozoites was elucidated by infecting a primary culture of human hepatocytes with *P. falciparum* and *P. vivax* sporozoites [24]. Though promising, this assay is rarely used as production of large number of sporozoites in insects is a rate-limiting step. Only few in vitro assays have been developed for hypnozoites of *P. vivax* and *P. ovale* and the monkey malaria parasite *P. cynomolgi* [25].

2.2.2. In vivo assays

Mouse models of malaria infection using rodent parasites are especially useful for studying the pathological effects of interactions between the host and the parasite. These models predict clinical outcomes of infections such as parasitemia, sequestration of parasitized red blood cells, splenomegaly, pulmonary edema and hematological and biochemical phenomena. Laboratory rodent parasites such as *P. berghei* and *P. yoelii* are used for evaluation of plant extracts and compounds [26]. With advances in genetic manipulation, humanized mouse models to study blood- and liver-stage *P. falciparum* infections in genetically modified mice have been recently reported [27]. These models have also been used in studying cell-mediated immune responses to liver-stage malaria vaccines [28]. Preliminary tests against *P. vivax* in non-human primate models like *Aotus* and *Saimiri* monkeys have also been carried out [29].

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2.2.3. Bioactivity-guided studies, compound isolation and identification

In common practice, traditional herbal remedies are prepared in water, either at room temperature or by boiling to obtain a decoction. Alcoholic solvents are also used as they produce higher extract yield and extract a wider variety of chemical components compared to aqueous extraction [30]. Separation and purification processes for antimalarial plant extracts and fractions involve different chromatographic methods. Frequently, as the extract is separated sequentially, antiplasmodial activity is monitored with a high-throughput in vitro bioassay until the compounds responsible for activity are isolated. This method is based on the assumption that antiplasmodial activity is limited to one or few compounds, whereas when such activity is due to different compounds acting synergistically, it may be lost with further separation [31]. Chromatographic procedures commonly employed include flash column, medium- and high-pressure liquid chromatography and centrifugal countercurrent chromatography. The structure of isolated compounds is determined on the basis of their spectroscopic properties using mass spectrometry, ultraviolet and infrared spectroscopy and complete proton and carbon mapping using one- and two-dimensional nuclear magnetic resonance techniques. It has also been possible to use tandem or hyphenated techniques of these spectroscopies for full stereochemical elucidation of constituents without isolation from extracts [32]. The compound obtained is thereafter subjected to further testing, extending to transmission and radical cure assays. Following the selection of a lead compound, it may be optimized by synthesizing chemical derivatives with the desired bioavailability, potency and selectivity before pre-clinical testing for efficacy and safety, preparatory for phase I clinical testing [33].

3. Isolated compounds and antiplasmodial activity

Some examples of identified compounds that exhibit good antimalarial activity *in vitro* are shown in **Table 1**. Criteria adopted for selection of the compounds shown were inhibition of *P. falciparum* growth by 50% at a concentration of either < 5 μg/mL or < 5 μM in vitro, with high selectivity (>100) for the parasite, where selectivity is expressed as

$$Selectivity\ (S) = \frac{EC_{50}}{IC_{50}} \tag{1}$$

where EC_{50} = effective concentration required to inhibit cellular growth by 50% and IC_{50} = concentration required to inhibit parasite growth by 50%.

From the compounds shown in **Table 1**, it is evident that a remarkable diversity of plant-derived compounds exists and they can form good templates for the design of novel antimalarials. One example of such is gedunin, a limonoid extracted from the leaves of *Azadirachta indica* with high antiplasmodial activity in vitro. Its antiplasmodial potency was attributed to the α,β-unsaturated ketone group in Ring A of its limonoid backbone, a 7α-acetate group as

well as its furan ring [34]. *Dichroa febrifuga* is a popular fever remedy in traditional Chinese medicine and guided studies led to the isolation and identification of febrifugine over 50 years ago [35]. Studies on febrifugine were hindered for a long time because of its toxicity. Some of its derivatives with good antiplasmodial activity have however been shown to exhibit lower toxicity compared with the parent compound [35]. The monoterpene indole alkaloid ellipticine and its isomer olivacine isolated from the bark of *Aspidosperma olivaceum* were shown to possess antiplasmodial effects in addition to its previously reported antitumor properties [36]. The selectivity of the ellipticine and olivacine for the parasitic targets was evident from the high *S* values (500–1200, ellipticine and 330–390, olivacine) against *P. falciparum* K1 and 3D7 [36]. Ellipticine was shown to be antiplasmodial, by inhibiting heme crystal growth and interacting with parasite DNA. It was speculated that this effect was similar to that of other analogous cryptolepine- and harmane-type indole alkaloids, depending on structural similarity [36]. *Uvaria leptocladon* is a shrub growing in the West Usambara Mountains of Tanzania, where it is used against cerebral malaria [37]. Investigation of the Tanzanian *U. leptocladon* root bark afforded the isolation of the chalcones, uvaretin and diuvaretin, which were shown to possess antiplasmodial activity with $IC_{50} < 5$ μg/mL [38]. Antiplasmodial screening of *Dorstenia barteri* twigs yielded the isolation of the prenylated chalcones, bartericin A ($IC_{50} = 2.15$ μM) and 4-hydroxylonchocarpin ($IC_{50} = 3.36$ μM) which were devoid of toxicity to erythrocytes at concentrations below 20 mM [39]. The authors deduced that the presence of a hydroxylated prenyl group on carbon 5′ on Ring B of bartericin A enhanced its antiplasmodial activity compared to a prenyl group at the same position in 4-hydroxylonchocarpin [39]. Lanaroflavone is a biflavonoid isolated from the methanol extract of the aerial part of *Campnosperma panamense* Standl. In vitro screening revealed its highly selective ($S = 159$) antiplasmodial activity ($IC_{50} = 0.48$ μM) [40]. For this compound, it was observed that its C-4‴—O—C-8 interflavonoid linkage was relevant for antiplasmodial activity [40].

A study of species of *Carpesium* genus used as traditional remedies for the treatment of parasite infections led to the identification and isolation of ineupatorolide A, a sesquiterpene from *Carpesium rosulatum*. Of particular interest were its high antiplasmodial activity ($IC_{50} = 0.019$ μM) and selectivity ($S > 1000$) [41]. *Bowdichia nitida* Spruce ex Benth., commonly referred to as "sucupira," is distributed in the Brazilian Amazon and the seeds of this plant are traditionally used for rheumatic arthritis, fever and gouty arthritis [42]. 6α,7β-Diacetoxyvouacapane isolated from a methanol extract of *B. nitida* seeds displayed high activity against *P. falciparum* 3D7 (IC_{50} 0.39 μg/mL) and high selectivity for the parasite, as cytotoxic IC_{50} on COLO 201 cells was higher than 100 μg/mL [42]. Another antiplasmodial compound identified as neosergeolide, a quassinoid obtained from the root and stem of *Picralemma sprucei*, had high antiplasmodial activity (0.002 μM) and was cytotoxic toward selected tumor cell lines at concentrations ranging from 5 to 27 mg/mL [43]. Antiplasmodial activity-aided fractionation of *Piptadenia pervillei* leaves afforded the identification of (+)-catechin-3-gallate and (+)-catechin-5-gallate, which displayed high antiplasmodial effects against *P. falciparum* FcB1 and had no significant cytotoxic effects against the human embryonic lung cells MRC-5 [44].

Other compounds have also been investigated and found highly active against hepatic stage *Plasmodium* species. N-Cyclopentyl-tazopsine, a semisynthetic derivative of a plant-derived

morphinan compound, tazopsine, was shown to have specific activity against liver-stage parasites of *P. falciparum* (IC_{50} = 42.4 μM, *S* = 60) and *P. yoelii* (IC_{50} = 3.3 μM, *S* = 46) [45]. Its efficacy against hepatic-stage parasites indicates its potential for development as a prophylactic agent.

Compounds	Plant source	Family	Antiplasmodial activity	Source
Gedunin	*Azadirachta indica*	Meliaceae	(Pf D6) 0.039 μg/mL (Pf W2) 0.02 μg/mL	[34]
Febrifugine	*Dichroa febrifuga*	Hydrangeaceae	(Pf W2) 0.53 ng/mL (Pf D6) 0.34 ng/mL	[35]
Ellipticine	*Aspidosperma vargasii*	Apocynaceae	(Pf K1) 0.81 μM (Pf 3D7) 0.35 μM	[36]
Olivacine	*Aspidosperma olivaceum*	Apocynaceae	(Pf K1) 1.4 μM (Pf 3D7) 1.2 μM	[36]
Uvaretin	*Uvaria* spp.	Annonaceae	(Pf K1) 3.49 μg/mL	[37, 38]
Diuvaretin			4.20 μg/mL	
Bartericin A	*Dorstenia barteri*	Moraceae	(Pf W2) 2.15 μM	[39]
4-Hydroxylonchocarpin			3.36 μM	
Lanaroflavone	*Campnosperma panamense*	Anacardiaceae	(Pf K1) 0.48 μM	[40]
Ineupatorolide A	*Carpesium rosulatum*	Asteraceae	(Pf D10) 0.019 μM	[41]
6α,7β-Diacetoxyvouacapane	*Bowdichia nitida*	Leguminosae	(Pf 3D7) 0.97 μM	[42]
Neosergeolide	*Picralima sprucei*	Simaroubaceae	(Pf K1) 0.002 μM	[43]
(+)-Catechin-3-gallate	*Piptadenia pervillei*	Fabaceae	(Pf FcB1) 1 μM	[44]
(+)-Catechin-5-gallate			1.2 μM	

Table 1. Antiplasmodial compounds with high selectivity isolated from plants.

4. Isolated compound classes and intra-parasitic targets

4.1. Alkaloids

This group of plant secondary metabolites represents the largest group of plant secondary metabolites with the highest number of compounds displaying potent antiplasmodial activity. They also serve as good templates for synthesis of many quinolone-based antimalarial drugs. Alkaloids displaying potent antiplasmodial activity occur as steroidal alkaloids, bisbenzylisoquinolines, naphthylisoquinolines, indoloquinolines and indolomonoterpenoid alkaloids, among others. Quinoline alkaloids isolated from the bark of *Cinchona officinalis* including

quinine, quinidine, cinchonine and cinchonidine (**Figure 1**) are all highly effective against malaria, with cure rates exceeding 98% in humans [33]. Although quinine is associated with serious side effects, it has remained an important drug to treat severe malaria due to chloroquine resistance. Quinine is schizonticidal against all intraerythrocytic malaria parasites and gametocytocidal for *P. vivax* and *P. malariae*, but not against *P. falciparum* gametocytes [46].

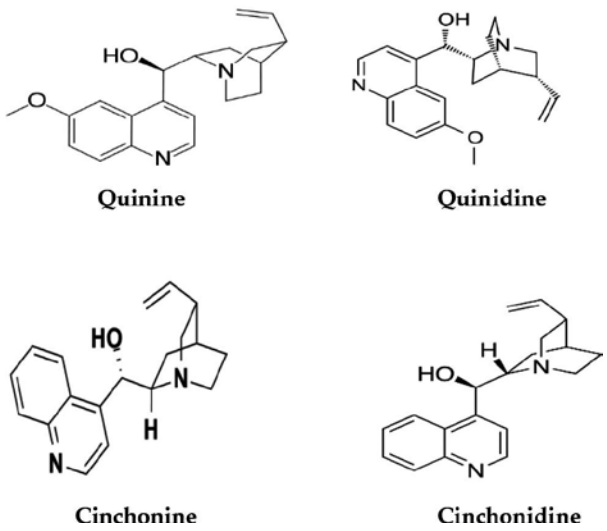

Figure 1. Chemical structures of cinchona alkaloids: quinine, quinidine, cinchonine and cinchonidine.

Although the mechanism of action of quinine has not been fully resolved, it has been reported to exhibit inhibitory effects on heme polymerization and heme catalase activity [47]. Following the success of quinine identification and use, natural antiplasmodial alkaloids have been isolated and reviewed by Kaur et al. [48]. Some alkaloids have been reported to inhibit fatty acid biosynthesis in the parasite [22], while some act as resistance reversers. The monoindole alkaloids strychnobrasiline and malagashanine isolated from Strychnos myrtoides potentiated the effects of chloroquine, quinolones, aminoacridines and halofantrine [49]. Malagashanine has no intrinsic antiplasmodial or cytotoxic action, but aids chloroquine accumulation in drug-resistant parasites by improving chloroquine influx and preventing its efflux from the parasites [50].

4.2. Flavonoids and chalcones

Flavonoids occur ubiquitously in many higher plants where they act as growth regulators and offer protection against plant pathogens [51]. They have been proposed to act by inhibiting the fatty acid biosynthesis (FAS II) pathway, which is present in the parasite's apicoplast but absent in human hosts [52]. The flavonoid, luteolin-7-O-β-D-glucopyranoside, was reported as the first natural product that targets plasmodial FAB I enzyme which regulates the FAS II pathway [53]. Some flavonoids have also been shown to inhibit L-glutamine and myoinositol influx

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into infected erythrocytes or act by interfering with hemin degradation [54, 55]. In addition, chalcones have been proposed to act by inhibiting cyclin-dependent protein kinases and plasmepsin II [56].

4.3. Terpenes and terpenoids

In recent times, attention has been devoted to this class of compounds especially sesquiterpenoids, following the discovery of the endoperoxide sesquiterpene lactone; artemisinin. These compounds are attractive because some possess intrinsic antiplasmodial activity and offer good starting points for chemical modification into derivatives with desired physicochemical properties and enhanced efficacy. Artemisinin and its derivatives owe their antiplasmodial effects to the presence of an endoperoxide bridge that generates toxic-free radicals when it is broken down (**Figure 2**). Another example of a highly potent antiplasmodial sesquiterpene is ineupatorolide A (**Table 1**).

Apart from the major classes of isolated compounds discussed above, other examples such as xanthones, stilbenes, coumarins, lignans, tannins and steroids have also been reported to exhibit potent antiplasmodial effects [57].

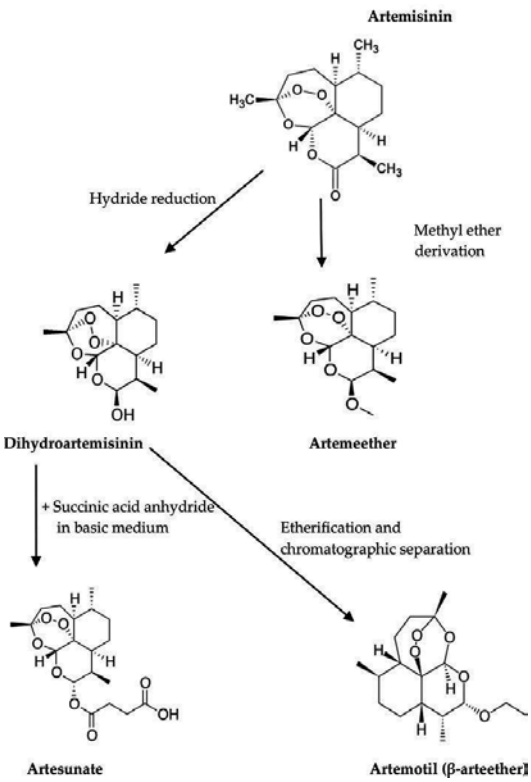

Figure 2. Artemisinin and its chemical derivatives in clinical use.

5. Clinical studies

Literature search revealed only few plant-derived extracts or compounds undergoing clinical studies and these are shown in **Table 2**. Spirotetrahydro β-carbolines (spiroindolones) present a unique group of compounds that share structural similarities with strictosamide, an iridoid indole alkaloid identified in an extract of *Nauclea pobeguinii* stem bark, but also present in *Nauclea latifolia* and *Nauclea officinalis* extracts [58, 59]. A spiroindolone (NITD609, **Figure 3**) displayed low IC_{50} within the range of 0.5–1.4 nM, showed no evidence of diminished potency against drug-resistant strains and was not significantly cytotoxic to mammalian cells [60, 61]. It was also effective against clinical isolates of *P. falciparum* and *P. vivax* (IC_{50} < 10nM) and comparable to artesunate. Additionally, it inhibited gametocyte development in vitro and oocyst development in mosquitoes [60]. *Argemone mexicana* decoction administered orally also produced antimalarial effects that were comparable to artesunate-amodiaquine combination in patients [62].

Plant extract/compound	Stage of clinical development	Mechanism of action
Nauclea pobeguinii [58]	Phase IIb/III	Not known
Argemone mexicana [62]	Phase IIb/III	Not known
NITD609 [60, 61]	Phase IIa	Chemotherapeutic, transmission blocking

Table 2. Plant extracts and natural product–derived compound in clinical development.

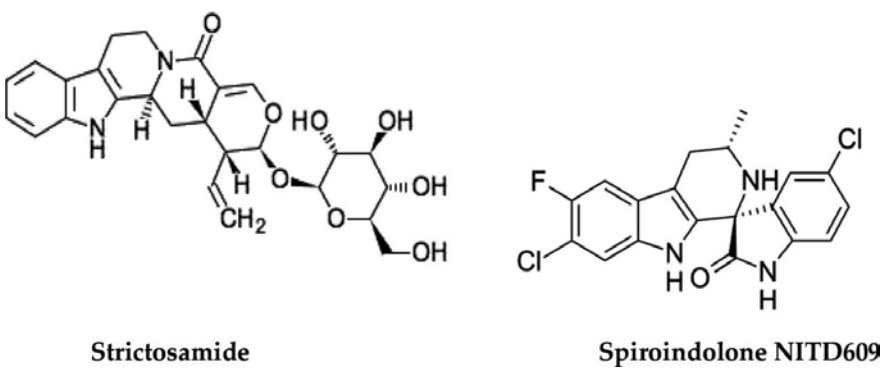

Figure 3. Chemical structures of strictosamine, an iridoid indole alkaloid and structurally similar spiroindolone in clinical development, NITD609.

6. Reverse pharmacology: from bedside to bench

Conventional drug discovery and development is an energy-, time- and resource-demanding venture; hence, the entire process results in minimal success rates. Millions of compounds are

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involved during initial screening and identified hits are ranked based on potency, ease of synthesis, known limitations to therapeutic use and novelty to determine a possible lead compound [33]. The lead compound is thereafter subjected to preclinical tests and various optimization processes to confer desired chemical and pharmacokinetic properties on it before final clinical testing. After passing through rigorous Phases I-III trials, it may be accorded statutory approval for clinical use. This is very expensive and time-consuming and many pharmaceutical companies are looking for new approaches in drug discovery that will lead to expedited launch of new, effective and safe drugs.

Reverse pharmacology is a science that integrates well-documented clinical experiences and observations toward lead development, through interdisciplinary studies (preclinical, clinical) for drug development [63]. Here, "safety" is the starting point as well-documented evidence of traditional use as medicine. This provides an important basis for further scientific testing. Hence, reverse pharmacology adopts a "bedside to bench" approach, compared to conventional "bench to bedside" drug discovery and development.

The use of *Artemisia annua* as treatment for fever and malaria in traditional Chinese medicine afforded the discovery of artemisinin through a reverse pharmacology approach. Today, artemisinin derivatives like artemether, artesunate, artemether and dihydroartemisinin remain useful antimalarial agents against drug-sensitive and drug-resistant malaria. However, the case of artemisinin is a unique one where artemisinin was identified as an active molecule, as not all traditional medicinal herbal extracts owe their therapeutic effects to a single chemical entity. The effects of some extracts may be due to different phytochemicals acting on different targets or a synergistic effect between different constituents and further separation and purification may lead to a loss of activity [30]. In this case, bioactivity markers should be identified and a standardized formulation of the extract should be prepared and screened using a systems biology approach before consideration for further development.

An example of antimalarial drug development using the reverse pharmacology approach in recent times is seen in the study of a standardized extract of *A. mexicana* for the treatment of malaria [64]. Initially, the authors conducted a retrospective treatment-outcome study to select a candidate for development before a dose-escalating clinical study to identify and choose a dose with desirable safety and efficacy. Next, they carried out a randomized controlled trial for comparison of the selected phytomedicine with conventional first line antimalarial therapy followed by identification of active compounds which could be employed as chemical markers to standardize the phytomedicine. This scheme was used successfully and can be adopted for antimalarial drug development (**Figure 4**). The process of identifying chemical and/or biological markers of efficacy which are used to ensure herbal medicine quality is known as standardization. This is an important step in drug development from herbal medicines, as quantity and quality of secondary metabolites depend on intrinsic factors, environmental factors and biotic factors [65].

It is interesting to note that subsequent to the report on the clinical efficacy of *A. mexicana* [62], three antiplasmodial protoberine-type alkaloids were isolated by conventional methods from an extract of *A. mexicana*, namely, berberine, allocryptopine and protopine, but berberine was

found to be cytotoxic relative to the parent extract, while allocryptopine and protopine were more selective for parasites compared to berberine [66] (see **Figure 4**).

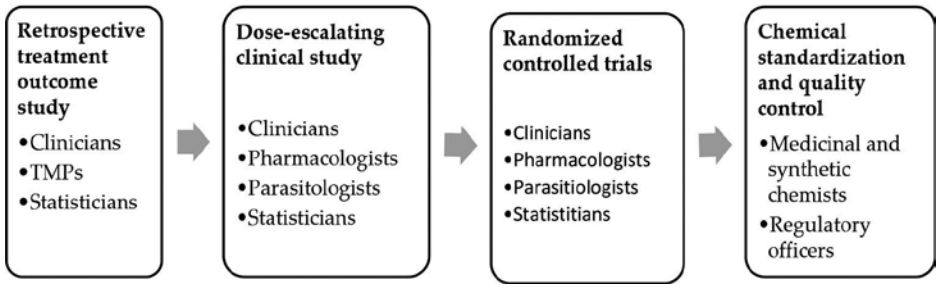

Figure 4. Antimalarial drug development by reverse pharmacology flow chart as described by Willcox et al. [64], showing key personnel involved during each stage of the process.

7. Conclusions

Naturally occurring antiplasmodial compounds in plants show vast chemical diversity but also exist within a complex mixture of other plant secondary metabolites which in itself constitutes a major challenge to efforts in identifying compounds responsible for biological effects. Other problems with plant-based drug discovery process range from the basic ones like sustainable access to plant material, seasonal and environmental variations and legislative issues concerning plant use, to challenges concerning complex fractionation procedures, small quantity of pure compounds and poor pharmacokinetic/physicochemical properties that negatively affect druggability [67]. With an increasing understanding and use of genomics, it is possible that bioactive molecules can be produced more efficiently using plant-cell cultures and genetically modified microbes [68]. This has already been exploited in the production of artemisinin precursors from genetically modified *Saccharomyces cerevisiae* and *Escherichia coli* [69].

Innovative drug discovery through reverse pharmacology or conventional methods especially in resource-constrained remote areas where medicines are urgently needed should be given more attention. There is the need to explore other aspects of the use of plant extracts and compounds as efficacy boosters or drug resistance reversers in combination with conventional therapy [50]. Efficacy screening against the parasite at all stages of development including gametocytes and hypnozoites should be incorporated in preclinical drug testing as they are often overlooked yet useful tools to identify agents that block transmission of resistance and prevent relapse [70]. In the course of literature review, a number of antimalarial compounds reported also displayed significant cytotoxic effects on human cells. Thus, screening for inhibitors against parasite-specific targets in organelles like the apicoplast and pathways such as heme degradation and type II fatty acid biosynthesis would likely identify active leads with highly selective antiplasmodial action.

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Chapter 11

Inactivation of Malaria Parasites in Blood: PDT vs Inhibition of Hemozoin Formation

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Céline Frochot and Samir Acherar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65053

Abstract

Malaria causes hundreds of thousands of human deaths every year, and the World Health Assembly has made it a priority. To help eliminate this disease, there is a pressing need for the development and implementation of new strategies to improve the prevention and treatment, due in part to antimalarial drug resistances. This chapter focuses on two strategies to inactivate the malaria parasite in blood, which are photodynamic therapy (PDT) and inhibition of hemozoin formation. The PDT strategy permits either a control of the proliferation of mosquito larvae to develop some photolarvicides for the prevention or a photoinactivation of the malaria parasite in red blood cells (RBCs) to minimize infection transmission by transfusion. The inhibition of hemozoin formation strategy is used for the development of new antimalarial drug by understanding its formation mechanism.

Keywords: hemozoin, photodynamic therapy, blood decontamination, heme-drug interaction, preventive treatment, curative treatment

1. Introduction

Malaria in humans is an infectious disease caused by parasites of the genus *Plasmodium*, and it is spread to humans by the bite of the female anopheles mosquito. Among the species of *Plasmodium*, five are capable of inducing human disease. These are the species: *falciparum*, *vivax*, *malariae*, *ovale*, and *knowlesi*. The first is the most widespread and the most virulent, which is responsible of 80% of infections and about 90% of deaths, especially in Africa.

In 2000, malaria was seen as one of the most critical constraints on global development and considered as a priority challenge of the "Millennium Development Goals" (MDGs). The main objective was to halt and begin to reverse the incidence of malaria by 2015 (Target 6C). The *World Malaria Report 2015* written by the World Health Organization (WHO) summarizes advances that have taken place in each WHO region over the 2000–2015 period [1]. Malaria is endemic in 95 countries mainly in Africa (88%). This report shows that this goal was achieved with an almost 37% and 60% drop in malaria incidence and in death rates, respectively, over this period. In 2015, 214 million cases of malaria were recorded including 438 000 that have led to the death of the patients, reflecting a decline of 18% and 48% in cases and deaths, respectively, over the 2000–2015 period. In May 2015, the *Global Technical Strategy for Malaria 2016–2030* was endorsed by the World Health Assembly. This strategy has its goal to reach a 90% reduction in global malaria incidence and mortality by 2030.

With regard to preventing malaria in countries at risk, the WHO recommends sleeping under an insecticide-treated mosquito net (ITN) and protecting by indoor residual spraying (IRS). Furthermore, the recommended treatment is an artemisinin-based combination therapy (ACT).

Despite a slight decrease, this disease remains a leading cause of death of children in Africa due in part to antimalarial drug resistances. Declines in cases and deaths caused by malaria are due to the development of new strategies such as the use of photodynamic therapy (PDT) for the control of the infection vector or to induce inactivation of *Plasmodium falciparum* and targeting the hemozoin inhibition, with the aim of preventing and treating malaria.

2. Inhibition of hemozoin formation

2.1. Generalities on the hemozoin production by *P. falciparum*

Hemoglobin, the main component of red blood cells (RBCs), represents almost 95% of the protein part of the cytosol (liquid fraction of the cell cytoplasm) up to reach 5 mM concentration in the cytoplasm (>300 mg/mL) [2]. Hemoglobin essential for cellular respiration is composed of a protein portion (globin) and a complex molecular structure centered on an iron atom (heme, ferriprotoporphyrin IX, Fe(II)PPIX which carries oxygen, and carbon dioxide from breathing).

During its life cycle in the red blood cell (RBC), the human malaria parasite (**Figure 1**), *P. falciparum*, gobbles up between 60 and 80% of hemoglobin from the host cell cytoplasm [3] by using a cytostome (cell mouth) for the purpose of transporting it to its acidic digestive food vacuole (pH ≈ 5.0–5.4 [4, 5]). At this acidic pH maintained by means of an ATPase pump enabling activation of a proton gradient, the hemoglobin is degraded into amino acids that are used for the production of parasite proteins, thereby allowing the release of free heme which is toxic to the parasite [6–9]. The hemoglobin degradation mechanism was studied in detail, and it was shown that it implies enzymes (proteases) present in the food vacuole of the parasite such as two aspartic (plasmepsins I and II) and cysteine (falcipain) proteases [10–14].

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The heme detoxification is a crucial step for the survival and growth of the parasite [15]. Heme is assumed to generate the formation of reactive oxygen species (ROS), *via* the Fenton reaction catalyzed by its iron atom [16–18], and hydroxyl radicals that may lead to peroxidation of lipid membranes [19–21]. It was postulated also that specific heme-H_2O_2 reaction might produce free radicals [21] which may result in oxidation of lipids, proteins, and DNA [22, 23].

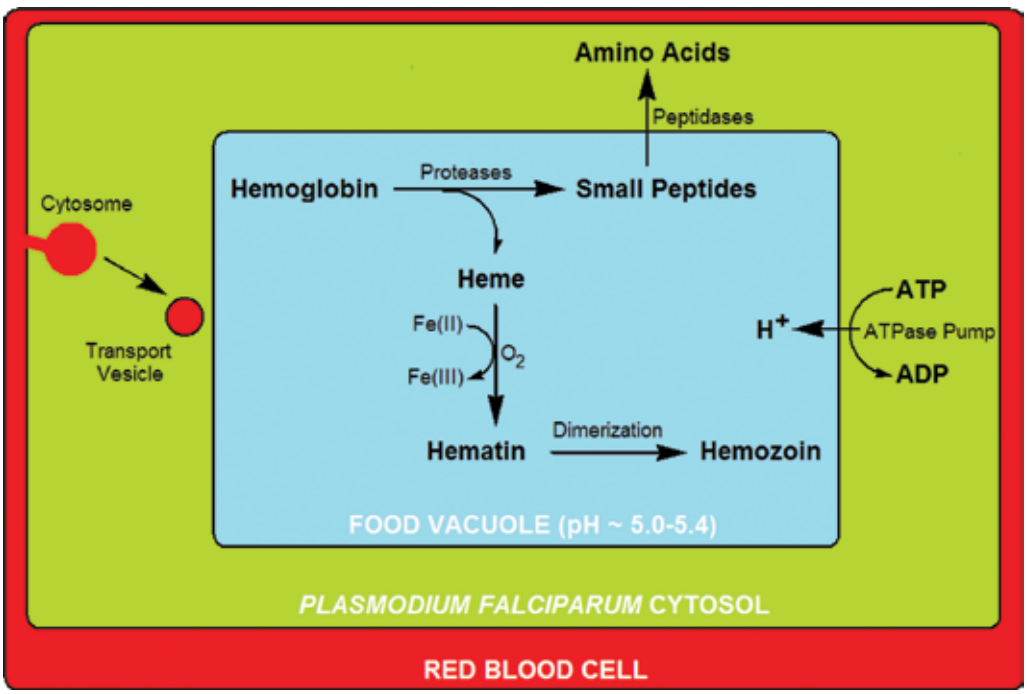

Figure 1. Hemoglobin degradation by *Plasmodium falciparum* in RBC.

The detoxification of heme begins with the self-oxidation of the Fe(II) in heme group into Fe(III) to form potentially toxic hydroxyferriprotoporphyrin IX (hematin, HO-Fe(III)PPIX; **Figure 2**) [8, 24, 25]. This detoxification ends with the formation of highly insoluble brown crystals known as hemozoin (malaria pigment; **Figure 2**) [26, 27] according to biomineralization or biocrystallization processes [28, 29] and not *via* a polymerization as previously believed; the x-ray structure is identical to a synthetic Fe(III)PPIX compound called β-hematin [30]. In 2000, the crystalline structure of β-hematin was determined to be a cyclic dimer of Fe(III)PPIX, involving two coordination bonds between the propionate side chain of one and the Fe(III) atom of the other [28]. These cyclic dimers are self-assembled in the crystal lattice *via* intermolecular hydrogen bonds which link the propionic acid side chains of each Fe(III)PPIX, thereby losing their toxic potential (heme detoxification), and then eliminated from the food vacuole. In 2010, the crystal structure of *P. falciparum* hemozoin has been solved by Klonis et al. after a reanalysis of x-ray crystallographic data for β-hematin [31].

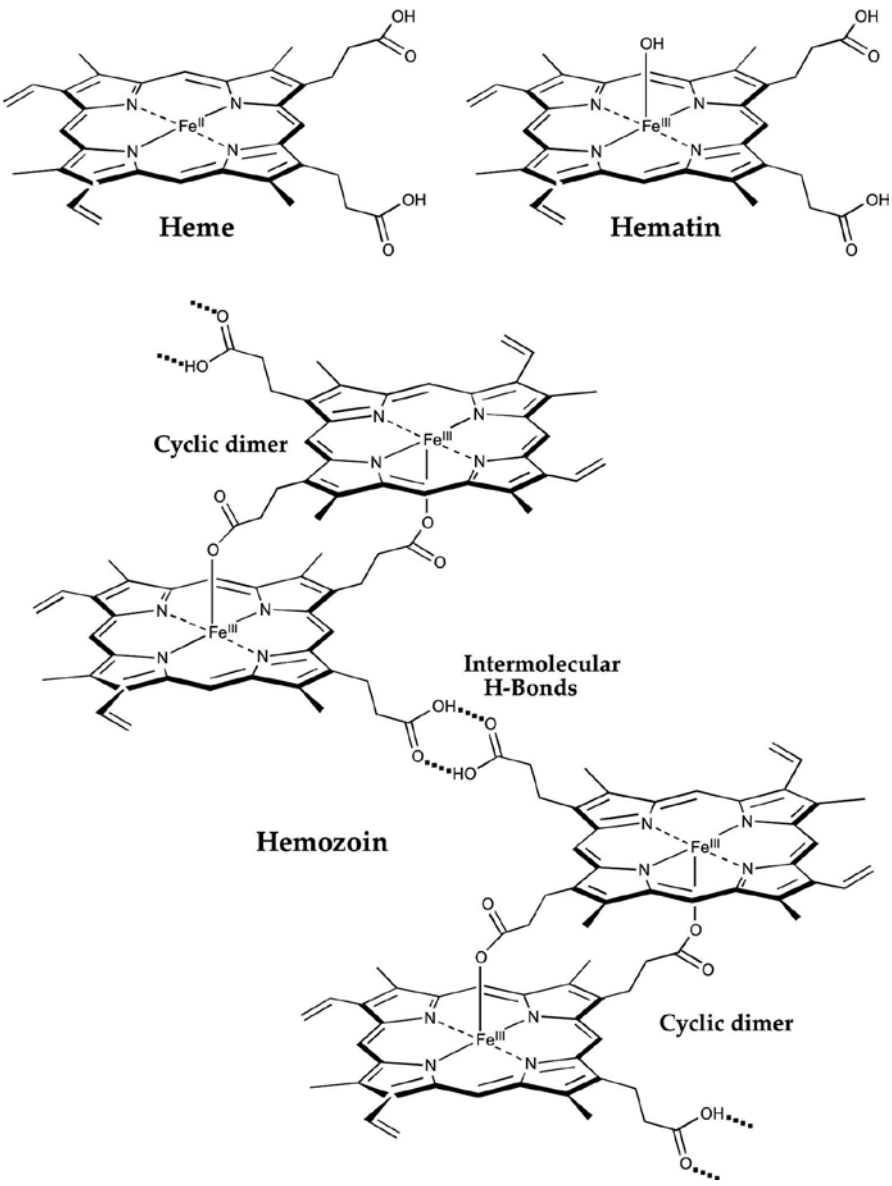

Figure 2. Chemical structure of heme, hematin, and hemozoin.

The mechanism concerning formation of β-hematin (hemozoin) in vivo and in vitro is still ambiguous and will be discussed in the following section.

2.2. Mechanistic assumptions about the hemozoin formation

The heme detoxification by *P. falciparum* results in the formation of hemozoin by template-mediated crystallization ("biocrystallization"), and a number of studies have been conducted

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in order to understand the hemozoin formation. Hemozoin or β-hematin (synthetic hemozoin) was used for these studies, and various mechanisms about the β-hematin formation have been postulated for the in vivo process.

Before beginning the discussion about mechanistic assumptions of the hemozoin formation, it is worth noting that when comparing the natural hemozoin and its synthetic version (β-hematin), we see a considerable difference in their size and shape. The natural hemozoin consists of small crystals ranging in size from 50 to 500 nm, whereas for the synthetic β-hematin, these crystals are bigger (50 nm to 20 μm) and depend on solvent used for the recrystallization. This difference in size can lead to diverse immunomodulatory responses [32].

The various studies of this mechanism gave rise to a number of assumptions [11, 33, 34] such as spontaneous [35, 36], autocatalyzed [37, 38], enzyme-catalyzed [39], lipid-catalyzed [40–43], and initiated or catalyzed by histidine-rich proteins (HRPs) [44–48], which can be divided into two main types: non-biological and biological conditions (**Figure 3**).

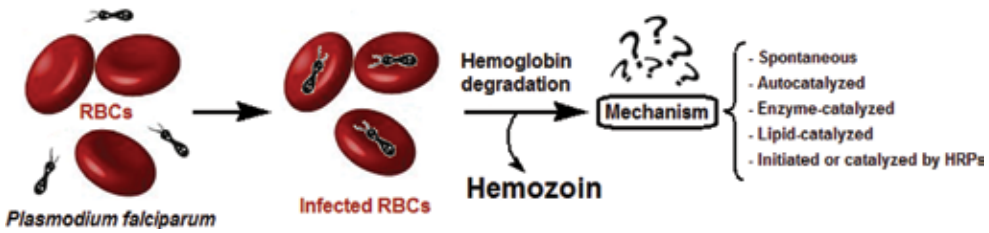

Figure 3. Postulated mechanisms about the hemozoin formation by *Plasmodium falciparum*.

The first category (non-biological conditions) is based on the assumption that β-hematin formation can happen spontaneously without any external help [35]. This observation comes from studies conducted in acetate solution, which shows that the β-hematin can be formed at a moderate low pH compared to the acidic digestive food vacuole [36].

The second category includes of all other mechanisms and provides a presumption that the β-hematin formation can catalyze itself or requires the presence of biological material (biocrystallization). The first idea about an autocatalytic process is, among other things, due to a recent observation of the continued growth of a preexisting hemozoin crystal [37].

As regards the second idea, it began in 1992 with the work of Slater and Cerami [39] which have shown that heme can react with trophozoite lysate extracts at pH 5–6 to generate hemozoin and that chloroquine, an antimalarial drug, can inhibit this formation. The authors concluded that the creation of the two propionate-Fe(III) linkages during the heme detoxification is catalyzed by an enzyme named heme polymerase. The use of extracts from *Plasmodium berghei* (rodent malaria) by Chou and Fitch gave equivalent results [49].

Despite being challenged, this heme polymerase theory attempted to explain hemozoin propagation without clarifying its initiation. This breach paved the way for other hypotheses about the formation of hemozoin involving a protein or enzyme [38]. Firstly, Hempelman in 2007 introduced the concept of "biocrystallization" instead of "polymerization" to describe

the hemozoin formation process [29]. One of the hypotheses suggested that biocrystallization is caused by enzymes, which postulate the presence of proteins such as histidine-rich proteins (HRPs). Sullivan and coworkers [48] showed that HRPs I, II, and III, present in the parasite's digestive vacuole, may be able to promote the formation of hemozoin in vitro. In 2008, Jani et al. [46] identified a novel heme detoxification protein (HDP) from *P. falciparum*, which is considered as one of the most powerful of the hemozoin-producing enzymes. As an example, we could cite the work of Choi and coworkers in 2002 and Nakatani et al. in 2014 concerning the elucidated reaction mechanisms of HRP II and HDP [44, 47]. These authors have shown that some histidine residues are active sites in these proteins and can bind with heme to promote the heme dimerization by bringing two molecules. This dimer would be used as a crystal growth initiator of hemozoin. Recently, Chugh and coworkers have established that HDP and falcipain-2 can work in tandem within the digestive food vacuole of the parasite to transform hemoglobin efficiently into hemozoin [45].

Finally, the last proposed mechanism is the biocrystallization catalyzed by lipids [40–43, 50]. These lipids, produced by the parasite after digesting the transport vesicles and trapped in its food vacuole, have been characterized with spectroscopic studies [7, 41] and known as a neutral lipid blend (NLB) and monopalmitoylglycerol (MPG). In 2007, Pisciotta et al. proved that Fe(III)PPIX can be processed into β-hematin through the action of these lipids with the yield of 80% or more [51] as assumed by Sullivan two years before [27].

The design and development of new antimalarial drugs first begin with the understanding of the mechanism of action of *P. falciparum* after invading RBCs and giving rise to hemozoin formation *via* the heme detoxification. Although this mechanism is not completely elucidated and still requires much work, these assumptions allow researchers to develop new strategies with a view to solving the problem of antimalarial drug resistance concerning chloroquine and artemisinin, the two most antimalarial drugs used to treat malaria.

By way of example, new strategies envisaged include the use of PDT (Section 3) in order to kill mosquito larvae (prevention Section 3.2) or to inactivate malaria parasites in the RBCs (treatment Section 3.3) but also the design of new antimalarial drugs that are able to inhibit the β-hematin formation by heme-drug interaction (treatment Section 4).

3. Photodynamic therapy for preventive and curative treatments

3.1. Generalities

The therapeutic effects of light are known since ancient times and were widely used in combination with natural substances for centuries in Chinese, Egyptian, or Indian civilizations for the treatment of numerous diseases such psoriasis, vitiligo, and rickets [52]. The integration of the concepts of "phototherapy" and then "photosensitivity" in modern medicine is much more recent, since it originated in the work of Niels Finsen, a Danish doctor who demonstrated in the 1890s the positive influence of light on the healing process (Nobel Prize for Medicine in 1903) [53]. However, the concept of exogenous photosensitizer (PS), that is to say, therapeutic

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molecule introduced for the specific purpose of interacting with light to generate the desired therapeutic effect, was introduced only a few years later, at the turn of the twentieth century by Raab and von Tappeiner as related by Spikes in a very good historical review [54]. In 1900, Oscar Raab, a student at the Department of Pharmacology of the University of Munich in the group of Hermann von Tappeiner, tried to characterize the influence of acridine on the development of *Paramecium caudatum* and *Plasmodium malariae*, a paramecium responsible for malaria. Very quickly, Raab noted that, according to the hour of the treatment and the weather conditions, the impact of administered acridine on the survival of microorganisms seemed extremely variable. He quickly demonstrated that the mechanism of cell death induced by acridine requires activation by light irradiation. Later, von Tappeiner identified oxygen as the third component (with the PS and light source) involved in photo-induced mechanism. He proposed the term "photodynamic therapy" (photodynamische Wirkung in the original) to define all therapeutic protocols involving these three elements [55]. von Tappeiner's team experimented eosin as PS to treat tumors in six patients, and some promising results were obtained [56]. Unfortunately, in this period, the concept did not arouse reactions on behalf of the scientific world in Western medicine [57].

In summary, PDT is an innovative medical treatment involving the concomitant action of three components that are photoactivatable molecule called PS, light of a suitable wavelength, and oxygen present in the biological medium. After light excitation of the PS and energy transfer from the excited PS to oxygen, reactive oxygen species are produced especially singlet oxygen ($^1O_2$) that can destroy cancer cells in proximity. It is interesting to notice that the PS itself is nontoxic and turns out to be toxic only with light. Light is also nontoxic by itself. The selectivity of action of PDT allows through a localized light radiation to eradicate tumor cells while preserving healthy cells. PS fluorescence properties are also an asset that is utilized to visualize the diseased tissue. The mechanisms are summarized in **Figure 4**.

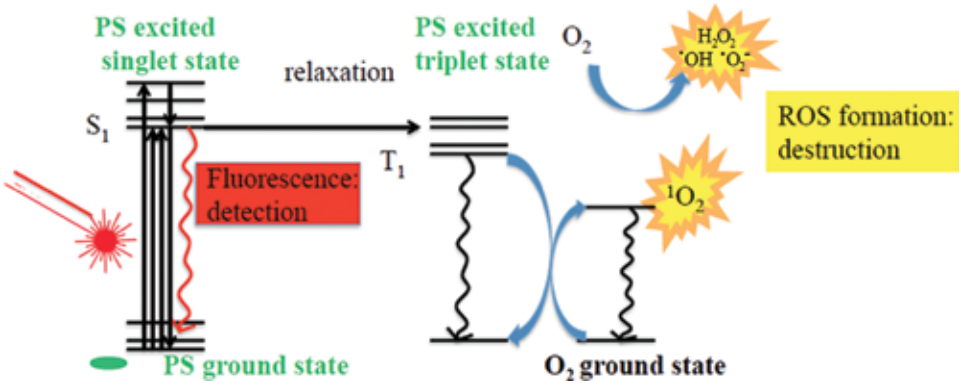

Figure 4. Mechanism of PDT (simplified Perrin-Jablonski diagram).

This technique was used clinically for many years, and in 1993, bladder cancer Photofrin PDT receives government approval in Canada. Since then, PDT has been developed in many countries of the world. PDT is an obvious treatment for dermatology applications, and it is

used daily for skin diseases such as actinic keratoses, acne, and wine stain [57]. PDT has been also widely employed as a treatment for age-related macular degeneration (ARMD). However, since 2006, intravitreal injections of Avastin, humanized monoclonal antibody having anti-angiogenic activity, significantly reduced the use of PDT to treat ARMD. In urology, the French company Steba Biotech has invested heavily to develop a new PS, the TOOKAD® (currently in phase 3) for the treatment of prostate cancer. The first clinical applications demonstrate the technical feasibility [58]. In gastroenterology, PDT demonstrated its effectiveness for the treatment of superficial cancers of the esophagus in patients ineligible for further treatment, with a postradiation recurrence, severe dysplasia in Barrett, and unresectable cholangiocarcinoma [59]. In gynecology, the interest of PDT has been shown in the treatment of cervical dysplasia of low- and high-grade cervical lesions [60]. Our team developed folic acid-targeted photosensitizers that could be very efficient to treat peritoneal carcinosis, and a preclinical evaluation is under progress [61, 62]. In pulmonology, the number of studies on the treatment of lung cancer is still limited, and the role of PDT in the therapeutic arsenal of the practitioner remains to be demonstrated. PDT appears to be a promising treatment for malignant pleural mesothelioma (MPM). Thus, PDT has been tested in phase I and phase II clinical trials to MPM patients in combination with extrapleural pneumonectomy or pleurectomy/decortication and an intravenous chemotherapy. The first work of the team of Professor Friedberg (University of Pennsylvania, Philadelphia, USA) has shown promising results with a median overall survival of 31 months [63]. PDT is not only a powerful technique to destroy human cells but also for viruses [64], yeasts [65], molds [66], bacteria [67], protozoa [68], parasites [69], and insects (Section 3.2). PDT is used in the development of new strategies to treat malaria and more generally to treat tropical diseases, either by controlling the propagation vector of the disease (Section 3.2), by inactivation of microorganisms responsible for these diseases, or by inactivating parasites (Section 3.3).

3.2. Prevention: destruction of mosquito larvae

3.2.1. Generalities

More than 700 million people are affected annually by mosquitoes in Asia, Mexico, Central America, South America, and Africa. A promising strategy to control diseases transmitted by mosquitoes (malaria, filarial, and dengue fever) is the control of these vectors. Mosquitoes are vectors of pathogens: *Aedes* is responsible for dengue fever, yellow fever, and encephalitis; *Anopheles* for malaria and encephalitis; and *Culex* for yellow fever and encephalitis [70]. Pesticides such as DDT (dichlorodiphenyltrichloroethane) have been used in affected area leading to decline of the mosquito population. Nevertheless, the use of these pesticides induces risks for safety reasons, development of resistance in major vectors, environmental and human health problems, etc. There is a need for developing improved insecticide, and the use of light with a PS is a possibility. In this case, the PS is called a photopesticide. One of the first researchers who described the potential of photosensitive molecules as insecticides was probably A. Barbieri in 1928 [71]. In 1979, rose bengal was used to treat Culex larvae [72]. In 1983, a review was written by J. Robinson about the photosensitizing dyes used as insect control agent [73]. The concept is to make a gulp down a small amount of PS to a mosquito larva, and then,

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after PS excitation by sunlight, the larva dies. Reviews have been published recently on this topic [70, 74]. The use of porphyrins has been described from the late 1980s [75, 76]. Different porphyrin derivatives have been then tested such as chlorophyllin, pheophorbide, and hematoporphyrin in laboratory conditions but also in semi-field conditions. A synthetic *meso*-substitute developed by Lucantoni et al. in 2012 had a potent photosensitizing activity on *Aedes aegypti* larvae that are responsible for the dengue in laboratory conditions [77].

3.2.2. Anopheles mosquitoes: the primary vector for malaria

Malaria is spread to humans by the bite of the female anopheles mosquito. In 2012, Fabris et al. described the photolarvicidal activity of a new PS called C12-porphyrin (5-(4-*N*-dodecyl-pyridyl)-10,15,20-tri(4-*N*-methylpyridyl)-21*H*,23*H*-porphyrin tetraiodide) [78]. This molecule was first supplied by Frontier Scientific Inc. (US Patent no. 6 573 258), and our team improved the synthesis and performed the photophysical properties study [79]. The structure of molecule is presented in **Figure 5**.

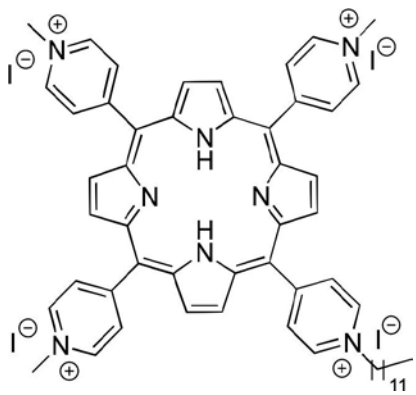

Figure 5. Chemical structure of C12-porphyrin.

In collaboration with the Institut de Recherche en Sciences de la Santé (IRSS) located in Burkina Faso, Fabris et al. studied the potential of C12-porphyrin as a photolarvicide for the control of *Anopheles*. Two different formulations with C12 were prepared: one is composed of Eudragit S100, an anionic methacrylic acid/methyl methacrylate copolymer, and the other is a fraction of cat food pellets. Both of them proved to be very efficient in laboratory conditions. The porphyrin-mediated photoinactivation of anopheles larvae could represent an interesting approach in the achievement of reduction of malaria morbidity and mortality.

3.3. Prevention: photoinactivation of parasites in blood

3.3.1. Generalities

With the emergence of many antibiotics, PDT declined for the treatment of parasite-related diseases, and it is only in recent decades that it knew a regain of interest with the increasing

problem of antibiotic resistance [80]. Antibiotic resistance is a global problem that reduces the power of conventional treatments of many diseases (both nosocomial and community-acquired infections). It concerns all pathogens including bacteria, fungi, and viruses.

To circumvent this bio-resistance, an attractive approach is PDT as non-antibiotic strategy to inactivate microorganisms (bacteria, viruses, parasites, etc.). This process is called antimicrobial photodynamic therapy (aPDT) [81, 82] or antibacterial PDT [83, 84] but is also known as photodynamic inactivation (PDI) [85–87] or photodynamic antimicrobial chemotherapy (PACT) [88–91]. This treatment can be effective in the case of chronic ulcers, infected burns, acne vulgaris, and a variety of local bacterial infections but also in the case of periodontitis [92], dengue [93], tuberculosis [94], viral infection [95], and malaria [96–99]. A very large variety of microorganisms have been studied and are listed by Alves et al. in a recent review [70] in which the insect pest elimination, water disinfection, and elimination of food-borne pathogens are described. A state of the art of PDT (potential) applications in animal models and clinical infectious diseases has been submitted by Dai et al. in 2009 [95], and numerous PSs are described [99, 100].

3.3.2. Inactivation of P. falciparum in human RBCs

Malaria is no more considered as poverty-related disease in Western countries, and attention has been paid to developing blood decontamination methods, vaccines, or new therapies. The spread of malaria disease, particularly with *P. falciparum*, in high-risk countries by blood transfusion is very worrying, especially as global traveling is continuously increasing. Inhabitants of tropical and subtropical regions where malaria is endemic can develop an immune response. However, they can carry a significant amount of parasites that can be transmitted by transfusion even if the blood is frozen (3 weeks' survival) [101, 102]. Decontamination of blood can be carried out according to several protocols including solvent-detergent methods, filtration, deleucocytation, photochemical techniques, etc. PSs such as psoralens, porphyrins, acridines, phenothiazines, porphyrins, and others can be used as additive for blood sterilization, and numerous protocols have been described, some of them could be found in [103, 104].

The life cycle of *Plasmodium* can be characterized by two phases: (a) the asexual proliferative phase in humans (intermediate host), called schizogony. This phase takes place in two different locations in humans and chronologically first within hepatocytes in the liver (exoerythrocytic cycle) and in circulating erythrocytes (RBC cycle). It also stands in the mosquito as a result of the sexual phase, (b) a sexual differentiation phase followed by asexual reproduction, called sporogony, which begins in humans and continues in the mosquito by the maturation of these in male and female gametes. As already mentioned, in erythrocytes, *P. falciparum* ingests 30–80% of hemoglobin, which is then digested in the food vacuole (an acidic organelle) and detoxified into hemozoin. This hemozoin itself could be a PS for killing *P. falciparum*, and Leblanc et al. [105] demonstrated that a simple irradiation of infected cultured RBCs by a near IR laser (800 nm) could induce a ~0.5 log reduction in parasitemia, but this is not enough for decontamination of blood.

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Historically, Ehrlich's group was the first to use methylene blue (**Figure 6**) as a PS [106] and Rounds et al. conducted a pioneering work on the photokilling potency of a ruby laser and methylene blue on cells infected by *P. lophurae* [107]. Since then, a wide variety of dyes have been explored. Merocyanine 540 was one of the first PSs used for the decontamination of blood [108] which reduced the concentration of parasites by 3 log when exposed to light. However, the overlapping between hemoglobin and the PS absorption made it not suitable for deparasitization.

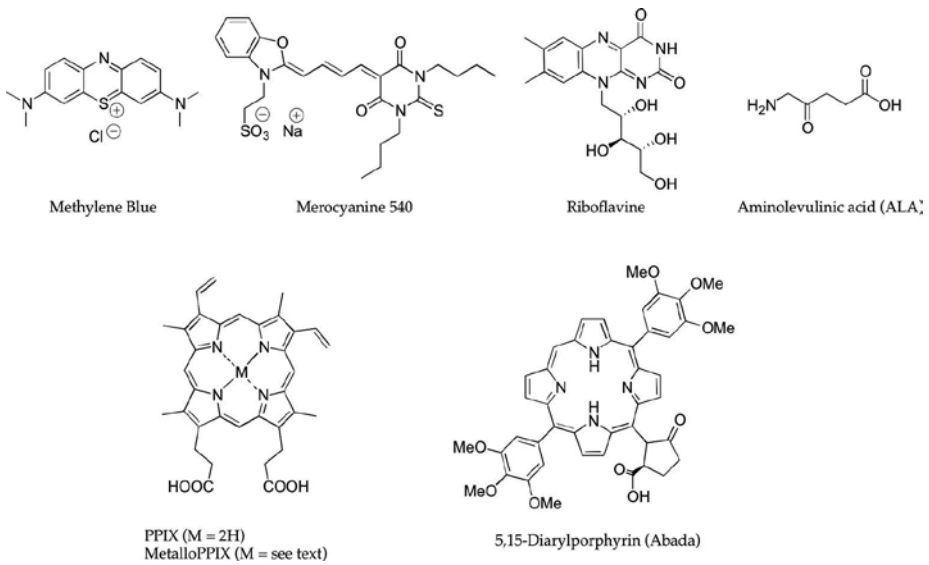

Figure 6. PS used for blood decontamination.

Riboflavin or vitamin B2 (**Figure 6**) deficiency is closely related to malaria [109, 110], and its administration can prevent hemozoin formation in the asexual cycle in the food vacuole of erythrocytes. Akompong et al. observed that addition of riboflavin can induce a 65% decrease of the food vacuole volume and subsequently damage to light-exposed contaminated blood [111]. In 2013, Goodrich's group tested the "Mirasol® pathogen reduction technology" (PRT) system against *P. falciparum* and *P. yoelii* [112]. This PRT system uses riboflavin and UV light for the destruction of a broad range of blood-borne pathogens and receives the European Community mark for both platelet and plasma applications. For *P. falciparum*, the percentage of parasitemia was 0.97 and <0.0005% before and after treatment, respectively. Similar results were obtained in vivo with blood of mice infected by *P. yoelii*. Recently, the "International Society for Medical Laser Applications" ordered a clinical trial named "Antimicrobial photodynamic therapy as a new treatment option for Malaria" in India on a group of 50 patients receiving an antimicrobial photodynamic treatment (riboflavin + 447 nm blue laser) over a period of 5 days plus conventional treatments [113].

In a recent research, Sigala et al. [114] demonstrated that sequencing of *P. falciparum* genome and some gene deletions did not affect the heme formation indicating that the host enzymes

are involved and can be a parallel pathway for the life cycle of *P. falciparum*. They showed the involvement of protoporphyrin IX (PPIX; **Figure 6**) in this parallel pathway and proposed a new treatment based on the chemoluminescence of luminol and aminolevulinic acid (ALA; **Figure 6**), which is the initial building block of PPIX [115] to produce ROS. The combination of ALA, luminol, and stimulating factor (4-iodophenol or dihydroartemisinin) decreased the parasitemia in the range of 75–80% [114]. ALA has also been described by Smith and Kain as a potentiate PS for killing *P. falciparum* in the presence of white light. The culture incubated by 0.2 mM ALA for 8 hours and exposed to light for 30 min exhibited a parasitemia less than 0.002% after 2 days [116].

PS	Conditions	Effects	Reference
Hemozoin	800 nm; 485 mW/cm^2; 60 min	~0.5 log reduction in parasitemia	[105]
Methylene blue	694 nm; 70 J/cm^2	Preferential uptake by infected erythrocytes by imaging	[107]
Merocyanine 540	485 nm; 26 W/m^2; 30 min	1000-fold reduction in parasitemia	[108]
Riboflavin	No irradiation /48h	65% decrease in food vacuole volume	[111]
Riboflavin	UV ; 6.24 J/mL ; 72 h	<0.002% survival	[112]
ALA	White light; 0.57 W/cm^2; 30 min	<0.0005% survival	[116]
ALA	Chemoluminescence by luminol	75–80% death	[114]
SnPPIX	No irradiation	IC_{50} = 6.5 μM (85 μM for chloroquine) on trophozoite lysate	[118]
Zn-PPIX	No irradiation	IC_{50} = 330 nM on RBC	[119]
Diarylporphyrin	No irradiation	IC_{50} = 20 nM on erythrocytes	[120]
Pheophorbide Ph4-OH	660 nm; 7 W/cm^2; 20 min	Total eradication with 2 μM/L	[121]
PC4 phthalocyanine	>600 nm; 60 J/cm^2; 10 min	<0.025% survival with 2 μM/L	[122]

Table 1. Bibliographic data.

In 1996, Martiney et al. [117] described a slight inhibition of hemozoin formation by using Zn-PPIX without light. Using trophozoite lysate of *P. falciparum,* Begum et al. obtained similar results with SnPPIX with an IC_{50} = 6.5 μM (to be compared to 85 μM for chloroquine) [118]. Recently, Garcia's group [119] encapsulated metal-PPIX (2H, Fe, Co, Cu, Mn, Ni, and Zn) in marine atelocollagen using the coacervation technique. They obtained an IC_{50} = 330 nM (for Zn-PPIX) on RBC and found that encapsulated Zn-PPIX was 80-fold more effective than the nonencapsulated Zn-PPIX and similar to chloroquine. In 2013, Abada et al. evaluated a series of 11 diversely substituted porphyrins against *P. falciparum* [120]. Only the 5,15-di-(3,4,5-trimethoxyphenyl)-10-(5-oxopyrrolidine-2(*S*)-carboxylate) (**Figure 6**) porphyrin has an

efficiency comparable to chloroquine with an IC_{50} value of 20 nM with a slight delay of infected mice survival.

The photosensitized inactivation of *P. falciparum* has been investigated by Grellier et al. [121] by using *N*-(4-butanol) pheophorbide derivative (Ph4-OH) as PS. Illumination at 660 nm (7 mW cm^{-2}) of parasitized whole blood induced a total eradication using 2 μM Ph4-OH and 20 min illumination, 4 μM Ph4-OH and 10 min illumination, or 8 μM Ph4-OH and 5 min illumination. The blood remained uncontaminated for at least 2 weeks. These results are better than those obtained with merocyanine 540 [108] and comparable to that obtained with phthalocyanines. In fact, Lustigman and BenHur [122] described the phthalocyanine HOSiPcOSi$(CH_3)_2(CH_2)_3N(CH_3)_2$ (Pc 4) as PS for blood decontamination and obtained an inactivation (≥99.8%) of *P. falciparum* clones 7G8 and HB3 by 10 and 40 min irradiation with a xenon short-arc lamp (>600 nm). The same team evaluated an IC_{50} of 24 nM in the dark [123]. The main results are summarized in **Table 1** (when available).

Besides the decontamination of blood or dialysis, numerous studies have been conducted to understand the physiology of the human malaria parasite *Plasmodium*, and some PSs have been used. For example, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY FL C_{16}) has been used as a marker for chloroquine resistance [124] or spatial distribution of oxidative stress in infected erythrocytes [125]. Other examples are 5,6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), 5′,6′-carboxy-10-dimethylamino-3-hydroxy-spiro[7*H*-benzo[c]xanthene-7,1′(3*H*)-isobenzofuran]-3′-one (SNARF), and 2,7-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF) for the measurement of the parasite's food vacuolar pH [126].

4. Curative treatment: drugs inhibiting β-hematin formation

Among various strategies, we will focus in the following part only on antimalarial drugs that inhibit the β-hematin formation by heme-drug interaction (purely π-π interactions). This strategy of drug development uses the heme scaffold itself as a hematin crystallization inhibitor (**Figure 2**). We can quote quinine, chloroquine, rufigallol and exifone and artemisinin, which are currently used as antimalarial drugs *via* this strategy (**Figure 7**).

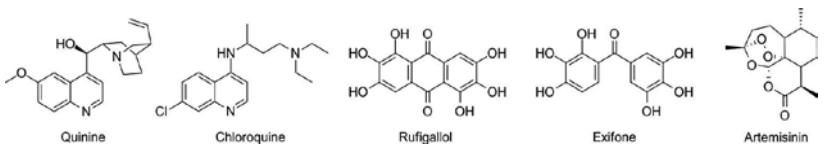

Figure 7. Structures of current antimalarial drugs.

Several studies and reviews [97] reported that porphyrins can inhibit the process of heme crystallization in the acidic food vacuole of the malaria parasite. As current antimalarial drugs,

porphyrins are able to inhibit the β-hematin formation by strong π-π stacking interactions. Several porphyrins have been studied for their use in heme aggregation inhibition.

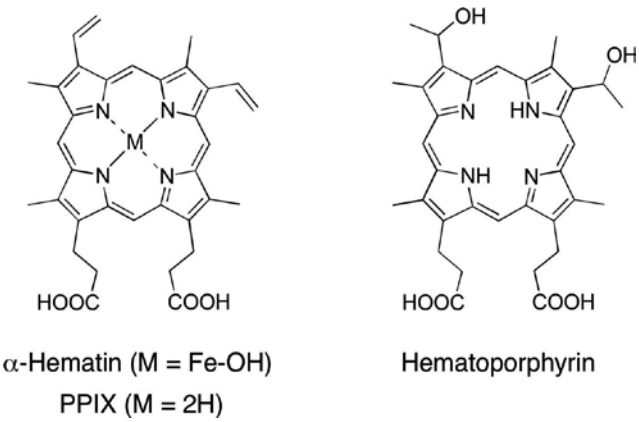

Figure 8. Structures of hematin, PPIX, and hematoporphyrin.

In 1997, Basilico et al. [127] evaluated the effect of two non-iron metalloporphyrins (PPIX and hematoporphyrin) on the crystallization of α-hematin (**Figure 8**) to β-hematin also called synthetic hemozoin (**Figure 2**). Crystallization of hematin may be achieved in 4.5 M sodium acetate buffer at 60°C [35]. Heme and β-hematin may be differentiated by their IR spectroscopic characteristics [128]. IR spectra of β-hematin show two bands at 1662 and 1209 cm^{-1}, which disappear in IR spectra of heme. From this property, Basilico et al. demonstrated that free-base porphyrins inhibit heme crystallization with hematoporphyrin more actively than PPIX. The presence of hydroxyl groups can explain the better inhibitory ability of hematoporphyrin.

In 1999, Tamarelli's team also showed that Fe(III)PPIX is reduced to Fe(II)PPIX as a novel endogenous antimalarial because Fe(II)PPIX molecules inhibit the crystallization process causing the death of the parasite [129].

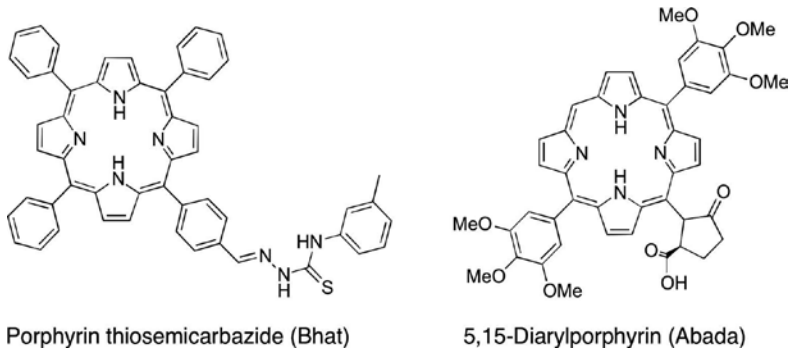

Figure 9. Structure of antimalarial drugs designed by Bhat (left) and Abada (right).

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Some researchers are interested in the synthesis of free-base porphyrins. In 2008, Bhat et al. [130] synthesized and evaluated the antimalarial activity of a series of porphyrin thiosemicarbazides. Only one compound (**Figure 9 left**) possesses an ability to inhibit β-hematin formation similar to chloroquine and quinine, the control drugs that are usually used in the malaria treatment. More recently, Abada et al. [120] synthesized a new 5,15-diarylporphyrin (**Figure 9 right**) with a good activity against *Plasmodium* with 20 nM IC_{50} value. The in vivo evaluation on *P. berghei* in mice model showed that this compound allowed delaying the death of the animal on about two days.

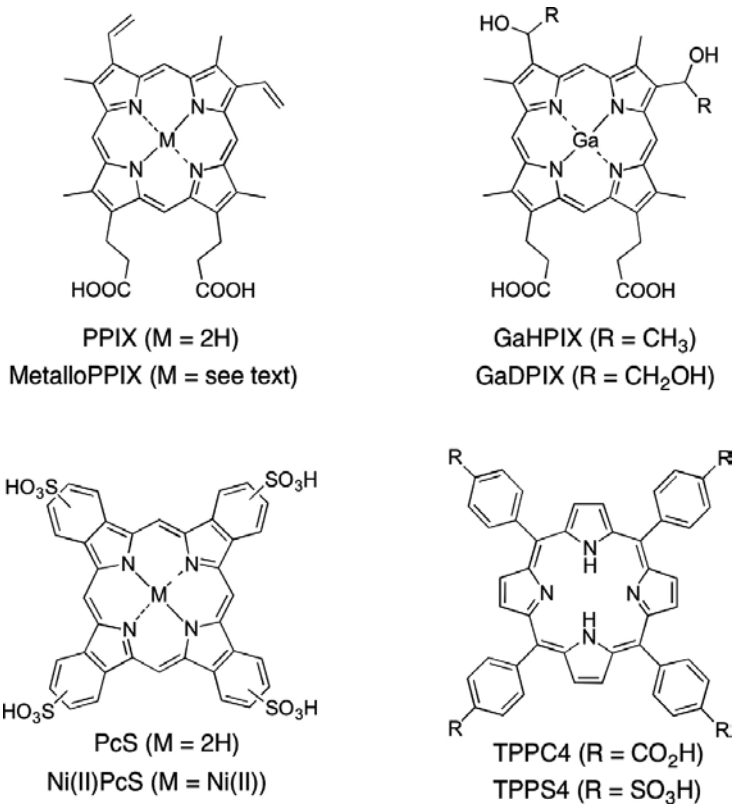

Figure 10. Structure of porphyrins and phthalocyanines developed by Wright and Begum.

In 2000, Wright's team highlighted the presence of other metal ions than Fe(III) can influence the conversion of heme to β-hematin. A number of metallo-PPIX, including Fe(III), Cr(III), Co(III), Cu(II), Mn(III), Mg(II), Zn(II), and Sn(IV) showed in vitro an ability to inhibit the β-hematin formation (**Figure 10**) [131]. In 2003 [132], phthalocyanines, phthalocyanine tetrasulfonate (PcS) and Ni(II)PcS, and anionic porphyrins, *meso*-tetra(4-sulfonatophenyl) porphyrin (TPPS4) and *meso*-tetra(4-carboxyphenyl) porphyrin (TPPC4), came to complete the previous study (**Figure 10**). All of them are inhibitors of heme crystallization. Among them, Mg(II), Zn(II), and Sn(IV) acted six times more efficiently than the free ligand PPIX and were more

efficient than the chloroquine standard as well. These results showed that metalloporphyrins with high oxidation state could form complexes with heme through the Fe-propionate linkages while being efficient crystallization inhibitors.

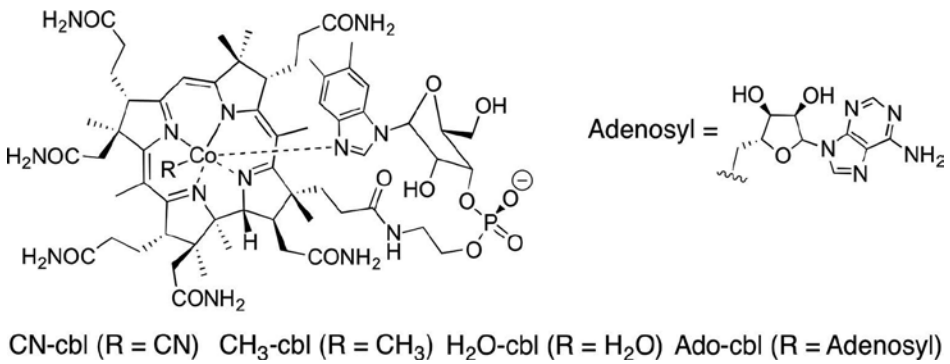

Figure 11. Structure of cobalamin derivatives.

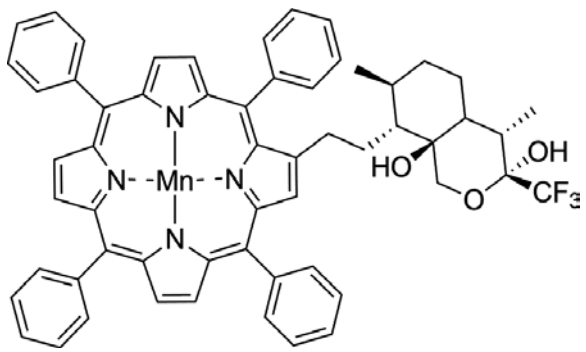

Figure 12. Mn(II) complexes of alkylated tetraphenylporphyrin with a fluorinated artemisinin.

The same behavior was observed by Begum et al. [118] who evaluated the antimalarial activity of free-base PPIX, deuteroporphyrin IX (DPIX), and hematoporphyrin IX (HPIX) and their corresponding complexes with Ga(III), Ag(III), Pd(II), Co(III), Mn(III), Sn(IV), Cr(III), and Fe(III) ions (**Figure 10**). Once again, SnPPIX at 15.5 μM had a better activity than the chloroquine control. Both GaPPIX and GaDPIX showed an antimalarial activity also.

In the same way, Chemaly et al. [133] observed that cobalamins (cbls) also called vitamin B12 (corrin ring with a chemical structure close to the heme but the central iron atom is replaced by an atom of cobalt) possess antimalarial activity. Methylcobalamin (CH_3-cbl), adenosylcobalamin (Ado-cbl), and aquacobalamin (H_2O-cbl) (**Figure 11**) showed increased efficacy over the chloroquine; cyanocobalamin (CN-cbl) was a little more efficient than chloroquine. The in vivo evaluation of vitamin B12 derivatives on the growth of *P. falciparum* (Ado-cbl > CH_3-cbl > H_2O-cbl > CN-cbl) was slightly lower than chloroquine or quinine.

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Rodriguez et al. [134] showed that Mn(II) complexes of alkylated tetraphenylporphyrin with a fluorinated artemisinin derivative (**Figure 12**) were effective inhibitors of β-hematin formation with an IC_{50} of 2.6 nM.

Benoit-Vical et al. [135, 136] showed a similar behavior with anionic metalloporphyrins. Alone the *meso*-tetrakis(4-sulfonatophenyl)porphyrin (TPPS) and *meso*-tetrakis(3,5-disulfonatomesityl)porphyrin (TMPS) complexed to manganese (**Figure 13**) inhibited slightly the β-hematin formation. However, the fact of combining them with β-artemether enhanced strongly the in vitro and in vivo antimalarial activity of β-artemether.

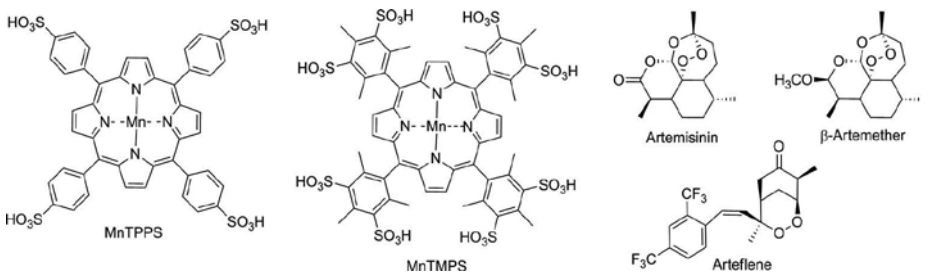

Figure 13. Structure of MnTPPS, MnTMPS, and other antimalarial derivatives.

5. Conclusion and perspectives

Malaria eradication is one of the great issues for humankind in the decades ahead. Based on figures from the *World Malaria Report 2015*, today more than ever, we are on the right track to reach this objective. The decline in cases and deaths caused by malaria stems from the relentless efforts of researchers to understand how the *P. falciparum* affects the RBCs. These different studies generated a wide range of strategies to prevent and treat malaria. Transfusion-transmitted malaria (TTM) must be understood as a high-risk situation, not only in African countries at risk but also around the world due to the increased immigration and travel from malaria-endemic areas. As mentioned in Section 3.3.2, malaria parasites can be transmitted by transfusion even if the blood is frozen (3 weeks' survival). In Europe, for example, all donated bloods are subjected to a large number of safety procedures including nucleic acid testing, blood filtration, or bacterial culture, but these are not done in many developing countries because of limited funds. All blood products are currently available in sterilized forms, except red blood cell (RBC) and platelet concentrates (PCs). The treatment of whole blood with a photosensitizer and light is a promising strategy. Very recent studies showed that this treatment can be achieved by riboflavin plus irradiation [137] and does not alter the quality of the blood [138]. As we already mentioned in Section 3.3.2, a first clinical study worldwide employing antimicrobial PDT is under progress in India using riboflavin as a photosensitizer and 447 nm blue laser. This chapter report focuses on innovative approaches using PDT or the design of new antimalarial drugs that is able to inhibit the β-hematin formation *via* heme-drug interaction.

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Chapter 12

Identification and Validation of Novel Drug Targets for the Treatment of *Plasmodium falciparum* Malaria: New Insights

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65659

Abstract

In order to counter the malarial parasite's striking ability to rapidly develop drug resistance, a constant supply of novel antimalarial drugs and potential drug targets must be available. The so-called Harlow-Knapp effect, or "searching under the lamp post," in which scientists tend to further explore only the areas that are already well illuminated, significantly limits the availability of novel drugs and drug targets. This chapter summarizes the pool of electron transport chain (ETC) and carbon metabolism antimalarial targets that have been "under the lamp post" in recent years, as well as suggest a promising new avenue for the validation of novel drug targets. The interplay between the pathways crucial for the parasite, such as pyrimidine biosynthesis, aspartate metabolism, and mitochondrial tricarboxylic acid (TCA) cycle, is described in order to create a "road map" of novel antimalarial avenues.

Keywords: malaria, *Plasmodium falciparum*, drug design, drug target validation, protein interference, metabolic map, oligomerization

1. Introduction

"Portrait of a serial killer," a commentary published in 2002 in Nature Journal states: "Malaria may have killed half of all the people that ever lived" [1]. Despite the effort and funds spent on malaria eradication, it continues to infect approximately 200 million people worldwide every year and kill one in every four infected [2]. While effective in the past, current antimalarials are becoming less and less reliable as the parasite rapidly develops drug resistance [3]. There

have been a number of extensive reviews covering the recent status of antimalarial research and parasite's resistance [3–11]. The shared message highlighted in these articles is that a constant supply of novel antimalarials is urgently required. Similarly to the Harlow-Knapp effect described for human kinase research [12], the majority of the antimalarial research is currently aimed at optimization of existing drugs targeting the known and validated pathways.

The currently used antimalarial drugs can be classified into few classes based on the mode of action [3, 7]. Briefly, the groups that receive the most attention of the researchers include the artemisinins and chloroquine-like compounds, which target the food vacuole and heme processing and detoxification [13, 14], antifolates targeting the mitochondrial dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), such as proguanil [15, 16], and mitochondrial inhibitors targeting the electron transport chain and consequently the pyrimidine biosynthesis. Unfortunately, resistance has been reported for nearly all available treatments [3, 7]. Unsurprisingly, compounds such as artemisinin and quinolines that target a broad range of essential pathways within the parasite have successfully been used for nearly 40 years before the widespread of resistance had been reported. In contrast, single-target drugs, such as antifolates and atovaquone, have lost their efficacy within few years of clinical use [11, 17]. A number of promising approaches to counter the fast emerging drug resistance suggested by Verlinden et al. include extension of combination therapy to three or more orthogonal drugs, development and use of multitargeting compounds interfering with unrelated targets, and deeper look into the unexplored alternative targets [3]. In all three cases, in order to successfully overcome the parasite's remarkable ability to develop resistance to nearly all drugs used against it, by far, a number of novel validated drug targets must be significantly expanded.

This chapter summarizes the pool of the mitochondrial and carbon metabolism targets that have been "under the spotlight" in recent years, as well as suggest a promising new avenue for the validation of novel drug targets. We will focus on the interplay between the pathways crucial for the parasite, such as pyrimidine biosynthesis, aspartate metabolism, and mitochondrial TCA cycle, in order to create a "road map" for further antimalarial drug development.

2. The Harlow-Knapp effect

A scientific analogue of biblical "The rich get richer and the poor get poorer" can be rephrased as "the propensity of the biomedical and pharmaceutical research communities to focus their activities, as quantified by the number of publications and patents, on a small fraction of the proteome" [12] or the "Harlow-Knapp effect." It was first noted by Harlow and colleagues [18] and further expanded by Knapp group [19], based on the analysis of the amount of publications and patents featuring human protein kinases. Kinases are known to regulate the majority of the cellular pathways including those involved in cancer and other diseases. It was observed that despite the availability of human kinome [20] more than three quarters of protein research was still focused on just 10 per cent of the kinases that were already known before the kinome publication [21]. Edwards and co-workers have also noticed that "the availability of research tools influences a protein's popularity." In other words, scientists tend to further explore the well-known systems, ignoring the less studied biomolecules where the probing tools are yet unavailable.

http://dx.doi.org/10.5772/65659

The availability of such tools for each system greatly limits the research opportunities and the attention to said system. Antimalarial research is not an exception to Harlow-Knapp effect: a limited opportunity for genetic manipulation [22] and complex life cycle of the parasite makes novel drug target validation highly challenging. Similarly to the human kinase research scientists tend to "keep looking under the spot light" among the few already validated targets, such as mitochondrial bc1 complex in malaria (target of the widely used Atovaquone), trying to optimize the existing compounds. Since first mentioned in the literature, there have been published more than 40 articles featuring plasmodial bc1 complex [23] and to the date it remains one of the most cited plasmodial enzyme.

Dihydroorotate dehydrogenase from *Plasmodium falciparum* (*Pf*DHODH) is another clear example of the Harlow-Knapp effect in antimalarial research. Since first proposed as a potential drug target more than a decade ago [24] and first inhibitors reported few years later [25], the major part of the research effort was focused on the optimization of the initial scaffold. In addition to the recent achievements in *Pf*DHODH inhibitor discovery by Phillips et al. [26], orthogonal methods, such as fragment-based drug design and virtual screening, have already yielded a number of very potent chemical scaffolds for this enzyme [27].

This divergent approach should be further exploited for other targets in order to yield novel and more potent scaffolds and support the antimalarial research.

3. Combinational therapy

The compound artemisinin and its derivatives have long been considered the most active and potent antimalarials for their efficacy against nearly all parasite stages [9, 14]. Artemisinins are believed to cause alkylation of proteins and heme and lead to oxidative damage within the parasite as well as affect the heme-related detoxification, although the exact mode of action is still a subject of debate [9, 14, 28]. Artemisinin-based combination therapy (ACT) is still recommended by World Health Organisation (WHO) for the treatment of uncomplicated falciparum and non-falciparum malaria in nearly all areas [7]. ACT implies the use of the fast acting artemisinin component, responsible for the rapid parasitemia clearance, in combination with another long-acting drug partner to eliminate the remaining parasites and suppress the selection of artemisinin resistance [29]. Despite the recent widespread of artemisinin-resistant falciparum malaria in Southeast Asia [30], the proven efficacy of combination therapy suggests that there is a pressing need for greater variety of highly effective antimalarial compounds. Combination of two or more drugs with different mode of action and resistance mechanisms significantly lowers the chances of the parasites to develop resistance to such treatment [31]. Thus, the research focus should be extended from optimization of existing compounds to development of novel research tools in order to explore and dissect other potentially druggable pathways of the parasite and thus bypass of the Harlow-Knapp effect. As stated by Verlinden et al.: "History has clearly indicated that new antimalarials must be continually developed in the ensuing event of resistance development to the current antimalarial arsenal." The occurrence of drug resistance in malaria is significantly faster than the development of antimalarials [3]. Thus, a constant supply of novel unrelated antimalarial compounds with orthogonal modes of action is urgently required.

4. The mitochondria as drug target for *P. falciparum* malaria

Mitochondria are organelles that act as the power plants of the cell, as they produce energy for all cellular activities. There are several molecular and functional differences between the mitochondria of *Plasmodium* species and those from the host. It is also known that the plasmodial mitochondria play a critical and essential role in the parasite's life cycle [5, 32, 33]. Previous studies have suggested that oxidative phosphorylation is not an essential pathway for parasite's survival during blood stage [34, 35]. In this stage, the parasite depends mainly on glycolysis as an energy source [36–38]. The observed glucose consumption in *P. falciparum*-infected red blood cells (RBC) was 75- to100-fold higher than in uninfected RBC [39]. Extraordinary glucose uptake during the infection leads to hypoglycemia, which together with an increased production of lactate and resulting lactic acidosis, are the major causes of mortality during severe malaria [40]. Thus, it is generally believed that the role of mitochondria in the parasite is not oxidative phosphorylation but the maintenance of the inner mitochondrial potential. Currently, the chemotherapeutic Malarone, a combination of mitochondrial *bc1* complex inhibitor Atovaquone and the dihydrofolate reductase inhibitor Proguanil, collapses the inner mitochondrial potential and induces parasite's growth arrest, confirming the mitochondrial metabolism to be crucial for the viability of the parasite. The importance of mitochondria for *Plasmodium* development in asexual stage is reinforced by the validation of another component of mitochondrial electron transport chain (ETC), dihydroorotate dehydrogenase (DHODH), as drug target [41, 42].

5. Electron transport chain (ETC)

The plasmodial mitochondrial electron transport chain (ETC) is composed of non-proton motive quinone reductases, such as dihydroorotate dehydrogenase (DHODH), malate-quinone oxidoreductase (MQO), glycerol 3-phosphate dehydrogenase (G3PDH), type II NADH dehydrogenase (NDH2, Alternative Complex I), and succinate dehydrogenase (SDH, Complex II), and proton motive respiratory complexes, including bc1 complex (Complex III), cytochrome *c* oxidase (Complex IV), and ATP synthase (Complex V) (**Figure 1**). The ETC requires ubiquinone (coenzyme Q) and *cytochrome c1* that function as electron carriers between the complexes [33, 44–47]. The (possible) roles of the ETC enzymes and their known inhibitors will be discussed in the following topics.

5.1. Dihydroorotate dehydrogenase (DHODH)

The *P. falciparum* enzyme dihydroorotate dehydrogenase (*Pf*DHODH) bridges the ETC and the pyrimidine biosynthesis; *Pf*DHODH catalyzes the key step of oxidation of dihydroorotate to orotate (a precursor for the biosynthesis of pyrimidine bases). The flavin mononucleotide (FMN)-dependent oxidation reaction catalyzed by DHODH can be divided in two half reactions: firstly, the oxidation of dihydroorotate through reduction of FMN and, secondly, the reoxidation of FMNH2 to regenerate the active enzyme. Two electrons resulting from this oxidation reaction are fed into the ETC through Flavin mononucleotide cofactor to

http://dx.doi.org/10.5772/65659

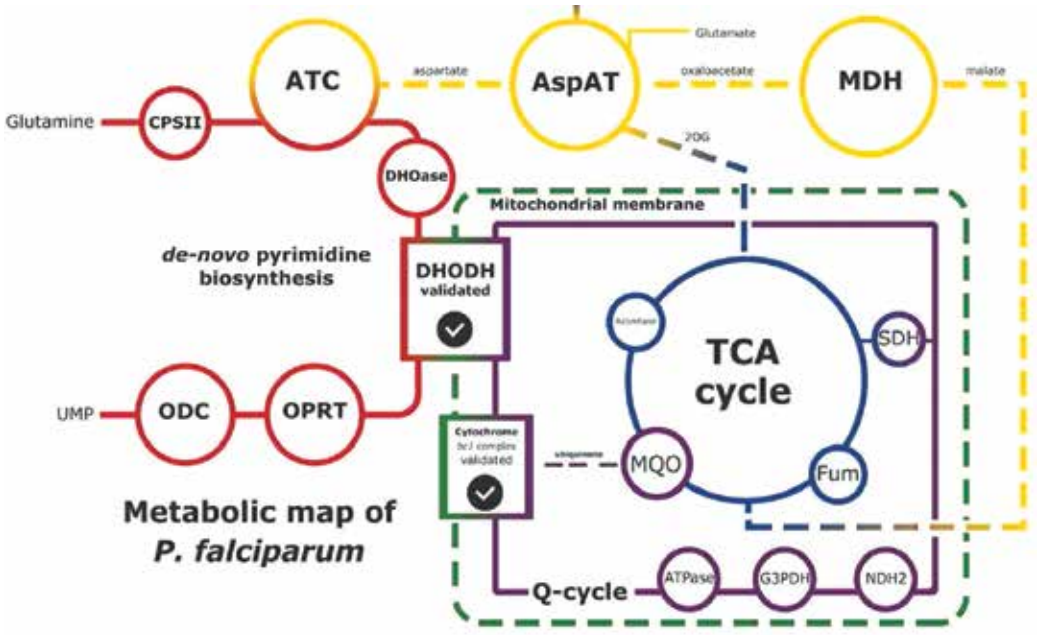

Figure 1. Suggested "roadmap" of essential metabolic processes of *Plasmodium falciparum* such as pyrimidine biosynthesis, aspartate metabolism, and mitochondrial TCA cycle. The map includes already-validated drug targets *Pf*DHODH [24] and cytochrome *bc1* complex [23, 43], as well as other promising targets.

ubiquinone, generated at the cytochrome bc1 complex, bridging pyrimidine metabolism and ETC [24, 48, 49]. Inhibition of *Pf*DHODH results in disruption of *de novo* biosynthesis of pyrimidines [48]. During the blood stage, the parasite depends strictly on this pathway for pyrimidine availability, which is essential for the formation of DNA, RNA, glycoproteins, and phospholipids [44].

Given the essential role of the *Pf*DHODH in the survivability of blood stage parasite and the significant differences to human DHODH [24], it is reasonable that the malarial enzyme has emerged as a novel validated drug target [26, 48, 50]. Inhibition of human DHODH was shown to be effective in treatment of autoimmune diseases, such as rheumatoid arthritis [51, 52]. The development of potent *h*DHODH inhibitors, such leflunomide and brequinar, led to the search of analogues with potential to inhibit plasmodial DHODH. These analogues were found to be poorly effective [53], potentially due to the differences in leflunomide and brequinar binding sites between human and plasmodial DHODH. These differences make *Pf*DHODH a potential species-specific drug target [24], which was extensively explored by a considerable number of studies. Although early research have not yielded effective results, the following studies have led to important achievements in the discovery of *Pf*DHODH inhibitors, such as benzimidazolyl thiophene-2-carboxamides [54–56], s-benzyltriazolopyrimidines [57], N-substituted salicylamides [58], trifluoromethyl phenyl butenamide derivatives [59], and triazolopyrimidine-based inhibitors [25, 60–64]. The triazolopyrimidine-based compound DSM265 was shown to be a potent inhibitor of the *Pf*DHODH and *Plasmodium vivax* DHODH

(*Pv*DHODH) with excellent selectivity versus *h*DHODH [48]. DSM265 has become the first DHODH inhibitor to enter the human antimalarial clinical trials, and preclinical development description was recently published, showing significant differences in DSM265 inhibitory activity between mammalian and plasmodial DHODHs. The kill rate of DSM265 for *in vitro* blood stage activity has shown to be similar to atovaquone, but significantly lower than observed for artemisinin and chloroquine. In addition, DSM265 has shown favorable pharmacokinetic properties, predicted to provide therapeutic concentrations for more than 8 days after a single oral dose in the range of 200–400 mg, what represents an advantage over current treatment options that are dosed daily. DSM265 was well tolerated in repeat dose, showed cardiovascular safety studies in mice and dogs, was not mutagenic, and was inactive against panels of human enzymes/receptors. Together, these data suggest that DSM265 has a high potential to be validated as a drug combination partner for either single-dose treatment or once-weekly chemoprevention [26].

5.2. Cytochrome bc1 (complex III)

The cytochrome bc1, also known as ubiquinol:cytochrome c oxidoreductase or complex III, is the only enzyme complex common to almost all respiratory ETCs [65]. This complex is composed of 11 different polypeptides, and its catalytic core is composed of three subunits, namely cytochrome b, cytochrome c1, and Rieske protein, also known as iron-sulfur protein (ISP) [66–68]. Cytochrome bc1 is found in the inner mitochondrial membrane and functions as a transporter of protons into the intermembrane space through the oxidation and reduction of ubiquinone in the Q cycle [67–70]. This enzymatic complex contains two distinct binding sites for the reduction and oxidation of ubiquinol and ubiquinone, both located within cytochrome b. The Qo site acts to oxidize ubiquinol near the intermembrane space, whereas the Qi site binds and reduces ubiquinone near the mitochondrial matrix [71, 72].

Although the crystal structure of plasmodial bc1 complex has not been solved, the high degree of sequence homology with other organisms of which the X-ray crystal structure is known (e.g. *Saccharomyces cerevisiae* [73]), allowed the discovery of many inhibitors. Cytochrome bc1 of *Plasmodium* is in fact a major drug target for the treatment and prevention of malaria and, to date, is the only component of the ETC with a clinically used antimalarial drug association [23, 43]. The compound atovaquone, a hydroxynaphthoquinone, inhibits cytochrome bc1 by binding to the Qo site. This inhibition leads to parasite death through the collapse of the *Plasmodium* mitochondrial membrane potential with no effect on the mammalian host [42, 74, 75]. Although atovaquone is a potent plasmodial bc1 complex inhibitor, its clinical utility is limited by the rapid emergence of resistant parasites when used as monotherapy [76]. Resistance to atovaquone has been developed due to mutations in the codon 268 (Y268S/C/N). These mutations affect the binding of the atovaquone to the target [77]. Because of that, atovaquone is used together with proguanil (Malarone) for treating uncomplicated malaria or as chemoprophylaxis for preventing malaria in travellers.

Aside of atovaquone, other bc1 complex inhibitors were described, as acridones [78], quinolones [79–81], pyridones [82, 83], and benzene sulfonamides [84]. Although many compounds have presented inhibitory potential against bc1 complex, this target might be considered

underexploited, since the majority of these compounds target the Qo site [85]. The Qi site of cytochrome bc1 has been far less explored and only the binding of a few compounds has been reported [86–89].

5.3. Type II NADH dehydrogenase (NDH2)

Instead of the canonical multimeric complex I, or NADH:dehydrogenase, found in mammalian mitochondria, the *Plasmodium* ETC possesses the type II NADH:quinone oxidoreductase (NDH2). This enzyme, also known as alternative complex I, is a five quinone-dependent oxidoreductase enzyme involved in the redox reaction of NADH oxidation with subsequent quinol production [90]. Although the activity of NDH2 is still not biochemically confirmed in *P. falciparum*, it has been described in some detail for other organisms that also possess the type II NADH:quinone oxidoreductase, such as plants, fungi, and bacteria [91–96]. Differently from complex I, NDH2 is not involved in the direct pumping of protons across the membrane. Instead of proton pumping, NDH2 enables the H+-unregulated generation of mitochondrial reducing power supplying the various respiratory chains with reducing equivalents from NAD(P)H [45, 90].

So far, no crystal structure of the *P. falciparum* NDH2 (*Pf*NDH2) is available, and prediction of *Pf*NDH2 is based on sequence and structural similarities to other redox enzymes [45, 91, 97]. Although reverse genetics of *Pf*NDH2 was shown to be not lethal [98], *Pf*NDH2 was described as a putative "choke point" in the mitochondrial ETC and has been highlighted as a potential target for antimalarial development [45, 90, 99]. Given the lack of structural data for *Pf*NDH2 and its poor homology to any other structure in PDB, the existing studies aiming to inhibit *Pf*NDH2 for "druggable" proposes have used chemoinformatics and virtual screening methods. *Pf*NDH2 (as other NDH2 analogues) has shown to be insensitive to rotenone, a well-known inhibitor of complex I [90, 100]. The compound 1-hydroxy-2-dodecyl-4(1H)quinolone (HDQ), initially identified as an inhibitor of yeast NDH2 [101], was reported to be a potent inhibitor of *P. falciparum* proliferation [102]. In fact, HDQ inhibits *Pf*NDH2 but, in addition, it disrupts mitochondrial function through the potent inhibition of the bc1 complex [103]. The compounds dibenziodolium chloride (DPI) and diphenyliodonium chloride (IDP) have also been reported to inhibit *Pf*NDH2 activity in crude lysate fractions and both have shown efficacy against whole parasite proliferation [90]. However, a further study put the potential of *Pf*NDH2 inhibition by these compounds into question, since the authors were unable to corroborate the previous findings through dose-effect profiles using purified recombinant *Pf*NDH2 [100]. These results suggest that DPI and IDP may not be effective inhibitors of *Pf*NDH2, but their antiparasitic effect might be attributed to other enzymes instead (*e.g. Pf*DHODH) [100]. Inhibition of *Pf*NDH2 by artemisinin has also been demonstrated, suggesting a dual role for mitochondria in the action of artemisinin [104]. More recently, Antoine et al. [105] demonstrated that the low degree of inhibition of this enzyme by artemisinin indicates a non-ETC mode of action.

In more recent efforts, Biagini et al. [81] undertook a high-throughput screen (HTS) against *Pf*NDH2 using HDQ in combination with a range of chemoinformatics as starting point. This approach led to the selection of the quinolone core as the key target for SAR, followed by the selection of CK-2-68 as a lead for further development [81, 106]. Structural alterations aiming to improve the inhibitory activity and aqueous solubility led to the

compounds SL-2-64 and SL-2-25, the last presenting activity against *Pf*NDH2 and whole-cell *P. falciparum* at nanomolar range. *In vivo* experiments using *Plasmodium berghei*-infected mice demonstrated that SL-2-25 was able to clear parasitemia in the Peters' standard 4-day suppressive test when given orally a dose of 20 mg kg^{-1} [107]. SL-2-25, as other quinolones in this study, had the ability to inhibit both *Pf*NDH2 and cytochrome bc1 at low nanomolar range, the same dual inhibition previously observed for HDQ. This dual targeting of two key mitochondrial enzymes suggests that the quinolone pharmacophore is a privileged scaffold for inhibition of both drug targets.

Although the recent efforts to inhibit NDH2 with antimalarial purposes have been a good improvement in the knowledge of its potential as a drug target, the report of *Pf*NDH2 crystal structure would allow a deep investigation on both biochemical characterization and drug design targeting *Pf*NDH2.

5.4. Mitochondrial glycerol-3-phosphate dehydrogenase (mG3DH)

Mitochondrial glycerol 3-phosphate dehydrogenase (mG3DH) is a ubiquinone-linked flavoprotein embedded in the mitochondrial inner membrane that transfers reducing equivalents directly from glycerol 3-phosphate into the electron transport chain [108, 109]. The *P. falciparum* genome has homologues of both cytoplasmic and mitochondrial G3DH and assays indicate that the addition of glycerol-3-phosphate stimulates electron transport through the inner membrane [110–112]. Together with NDH2, mitochondrial G3DH from *P. falciparum* (*Pf*mG3DH) is also suggested to play an important role in the redox balance under conditions of low O_2. Further studies might clarify the essentiality of mG3PDH in *Plasmodium* survivability and also evaluate its potential as a drug target.

5.5. Succinate dehydrogenase (SDH)

The succinate dehydrogenase (SDH), also known as succinate: ubiquinone oxidoreductase (SQO) or complex II, is an enzymatic complex involved in both TCA cycle, functioning as a primary dehydrogenase, and in mitochondrial ETC, functioning as electron donator [113]. This dual role makes SDH a direct connection between major systems in aerobic energy metabolism. The enzyme has been isolated and characterized from prokaryotic [114–117] and eukaryotic organisms [118–121], including *P. falciparum* [122, 123]. SDH is located in the cytoplasmic membrane in bacteria [124] and in the mitochondrial inner membrane in eukaryotes [125]. The enzymatic complex is highly conserved and is basically composed of four subunits: a flavoprotein subunit (SDH1) and an iron-sulfur subunit (SDH2) together form a soluble heterodimer that binds to a membrane anchor b-type cytochrome (a CybL (SDH3)/CybS (SDH4) heterodimer). In *P. falciparum*, the two major subunits possess molecular masses of 55 kDa (Fp, flavoprotein subunit) and 35 kDa (Ip, iron-sulfur protein subunit) [122]. The SDH activity has shown to be essential for *Plasmodium* survivability, what makes this enzyme an attractive target for antimalarial development. The already reported differences in kinetic properties between *P. falciparum* SDH (*Pf*SDH) and human SDH increase the probability that *Pf*SDH inhibitors might represent potent and selective antimalarial compounds [122]. In fact, SDH has shown sensitivity to a number of inhibitors, such as 5-substituted 2,3-dimethoxy-6-phytyl-1,4-benzoquinone

derivatives, plumbagin and licochalcone [125], but so far, inhibitors with potential for antimalarial development still have to be discovered.

5.6. Malate-quinone oxyreductase (MQO)

The malate-quinone oxidoreductase (MQO) is a peripheral membrane-bound flavoprotein, which catalyzes the oxidation of malate to oxaloacetate, reducing ubiquinone [126]. *Plasmodium* species possesses a group 2 MQO, in contrast to bacterial group 1 MQO [127]. *P. falciparum* MQO (*Pf*MQO) is part of both mitochondrial ETC and TCA cycle, substituting other mitochondrial malate dehydrogenases (MDH) [111, 112, 128]. To date, no crystal structure of the *Plasmodium* MQO or inhibition studies are available. However, recent experiments showed that while knockout of six enzymes of plasmodial TCA cycle did not cause any significant growth inhibition, no viable MQO-knockout strains of *P. falciparum* could be obtained yet [34]. These findings as well as the absence of MQO in the human host make the enzyme an interesting target for antimalarial drug discovery.

5.7. ATPase

Although malaria parasites generate most of their ATP through aerobic glycolysis during the blood stage of their life cycle, they appear to possess a complete ATP synthase complex [47]. *P. falciparum* ATP synthase (*Pf*ATP synthase) is not reported to generate ATP but is suggested to act as a proton leak for the ETC [46, 47]. The use of bedaquiline, TMC207, has been proven to be effective for the treatment of multidrug-resistant tuberculosis. This compound targets *Mycobacterium tuberculosis* ETC through inhibition of ATP synthase rising the hypothesis that this may also be a valid drug target for malaria in the future [129]. So far, only one *Pf*ATP synthase inhibitor was described. The compound almitrine, originally developed as a respiratory stimulant, has activity against *Pf*ATP synthase and at the cellular level [130]. Recently, a genetic study demonstrated that mitochondrial ATP synthase is dispensable in blood stage *P. berghei,* although is essential in the mosquito phase [131]. For *P. falciparum*, previous attempts to knock out the mitochondrial ATP synthase subunits were unsuccessful, suggesting an essential role played by this enzyme complex in blood stages of the parasite [47]. The difference in essentiality of ATP synthase between *P. falciparum* and *P. berghei* could be explained by a possible distinction in the requirements of the two species for ATP [131]. Still, more studies are needed to define whether or not ATP synthase is essential in *P. falciparum* blood stage and consequently evaluate its potential as antimalarial target.

6. Tricarboxylic acid (TCA) cycle

While *Plasmodium* relies mainly on glycolysis during the blood stage, the TCA metabolism does occur in asexual *Plasmodium*, but at low turnover [35]. The exact function of the plasmodial TCA cycle is still a subject of debate, as it does not seem to function like a conventional TCA cycle. In 2010, a branched TCA pathway has been suggested for the parasite [132] but further retracted [133]. It was proposed that plasmodial TCA enzymes function not only in the classical but also in the reverse direction, generating either reductive or an oxidative pathway,

depending on the direction. Both pathways would result in the generation of malate, which is subsequently exported from the mitochondria, with α-ketoglutarate (2OG) being antiported to feed both the oxidative and reductive pathways [132]. Depending on the nutrient availability, *Plasmodium* species might not excrete malate as metabolic waste, utilizing it for metabolic purposes [134].

Further metabolomic studies suggest that *P. falciparum* utilizes conventional TCA cycle during both sexual and asexual blood stages [35]. Functional respiratory chain appears to be essential for the maintenance of inner mitochondrial membrane potential as well as protein and metabolite transport within the mitochondrion. The authors have also reported an increased sensitivity of gametocyte stages to sodium fluoroacetate (NaFAc). NaFAc was previously reported to inhibit TCA cycle enzyme aconitase in *Leishmania* [135]. Both sexual and asexual cultures of *P. falciparum* treated with 1 mM NaFAc showed significant citrate accumulation in the parasite as well as decrease in downstream TCA metabolites, suggesting the specific inhibition of aconitase of *P. falciparum*. However, no significant growth inhibition of the asexual parasites was observed, while gametocyte development was significantly reduced. These findings provide a potential for future transmission-blocking therapy.

Recently, Ke et al. [34] reported significant flexibility in TCA cycle metabolism of *P. falciparum*. The knockout experiments with all TCA cycle enzymes showed altered substrate fluxes between mitochondrial and cytosolic pools in nearly all cases. Out of eight enzymes of the TCA cycle, knockout of six enzymes of the TCA cycle showed no detectable growth defects. However, the authors were unable to disrupt the genes encoding fumarate hydratase and malate-quinone oxyreductase, suggesting potentially essential role of these two enzymes in asexual parasite development.

Although the fully functional TCA cycle appears to be dispensable for parasite survival in asexual blood stages [34], the interplay of some TCA enzymes with other essential pathways still represents an interesting target for antimalarial drug development. Below, we describe the role of three enzymes (aspartate aminotransferase, malate dehydrogenase, and fumarate hydratase) in *Plasmodium* metabolism and also their potential for antimalarial drug discovery. Other enzymes involved in this pathway (e.g., *Pf*SDH, *Pf*MQO) were previously described within the ETC section (see above).

6.1. Aspartate aminotransferase

The enzyme aspartate aminotransferase (AspAT) catalyzes the reversible reaction of L-aspartate and α-ketoglutarate into oxaloacetate and L-glutamate. The AspAT from *P. falciparum* (*Pf*AspAT) was placed into the Ia subfamily, being the most divergent member of this group. The crystal structure of *Pf*AspAT reveals an architecture similar to that previously determined in the *Escherichia coli* (1B4X14–17) [136–139], yeast cytosolic [140], pig heart cytosolic [141], and mitochondrial and cytosolic chicken [142–144] homologues. *Pf*AspAT is a homodimeric enzyme [145, 146], and each subunit consists of a large PLP (cofactor) binding domain, a smaller domain, that shifts the enzyme from "closed" to "open" form in order to provide substrate binding and N-terminal region that stabilizes the interaction between the two monomers into a dimer [142, 147, 148]. Two independent active sites are positioned near

the oligomeric interface and are formed by residues from both subunits [146]. The active site is highly conserved between available AspATs, making the design of species-specific inhibitors very challenging. However, it is known that the active site requires the formation of a homodimer, and analysis of AspAT has highlighted the N-terminal region as being highly divergent from other AspAT family members in both sequence and structure [145, 146]. Such a divergence may allow a more specific interference with the parasitic AspAT oligomeric surfaces, which offers a unique opportunity to generate highly specific interference with protein function *in vivo*. Such an approach will be further discussed in this chapter.

6.2. Malate dehydrogenase

The enzyme malate dehydrogenase (MDH) catalyzes the reversible NAD(P)+-dependent oxidation of oxaloacetate to malate. Like other members of the NAD+-dependent dehydrogenase family, the MDHs possess two functional domains, the catalytic domain and the NAD+-binding domain. Protozoan MDHs are differentiated into two subdivisions: mitochondrial and cytosolic MDHs, the first being part of the TCA cycle, providing oxaloacetate for the generation of citrate and NADH to fuel the mitochondrial electron-transport chain. The mitochondrial MDH is absent in *P. falciparum*, being replaced by *Pf*MQO (described in ETC section). The cytosolic MDH is present in *P. falciparum* (*Pf*MDH) acting as a supplier of metabolites, such as malate, to the mitochondria and might be responsible for the generation of reducing equivalents to feed the respiratory chain [149].

The crystal structure of *Pf*MDH has recently been solved [150]. Analysis of *Pf*MDH structure revealed a tetrameric assembly, although isoforms of the enzyme from other species have been reported to be present as either dimers or tetramers. Similar to *Pf*AspAT, the oligomeric nature of *Pf*MDH and the low degree of evolutionally conservation of the oligomeric interface residues provide an opportunity for a highly specific protein interference approach (described further).

6.3. Fumarate hydratase

Fumarate hydratase (FH) is an enzyme that catalyzes the reversible conversion of fumarate to malate. Although *P. falciparum* contains a fumarate hydratase homologue (*Pf*FH), it differs substantially from the "class II" type enzyme found in yeast and mammalian cells [151, 152]. Instead, the *Pf*FH resembles the iron-sulfur-containing "class I"-type enzymes found in some bacteria and archaea [153]. *Pf*FH was shown to be essential to the asexual stages of the parasite [34]. *Pf*FH was initially suggested to be located within the mitochondrion [153], however, this localization is yet not entirely clear.

Fumarate is a side product of the purine salvage pathway and acts as metabolic intermediate of the TCA cycle. As previously mentioned, *P. falciparum* does not export fumarate as metabolic waste but converts the metabolite to aspartate through malate and oxaloacetate. Besides, *P. falciparum*-infected erythrocytes and free parasites incorporate labeled fumarate into the nucleic acid and protein fractions [153]. Taken together, these data provide a biosynthetic function for fumarate hydratase and suggest that this enzyme could therefore be targeted for the development of antimalarial chemotherapeutics.

7. Pyrimidine biosynthetic pathway

A key-step for spreading of malaria parasites in the human host is the extensive and rapid replication of parasite DNA, which depends on the availability of essential metabolites, such as pyrimidines [154, 155]. In the *Plasmodium* species, besides the DNA, the pyrimidine nucleotide is also involved in the biosynthesis of RNA, phospholipids, and glycoproteins [155–157]. Sequencing studies have revealed that, in malaria parasites, the genes encoding for the pyrimidine biosynthetic pathway enzymes have been conserved, whereas those responsible for pyrimidines salvage have not [158]. It means that, while human cells are able to acquire pyrimidines either through *de novo* synthesis or by salvaging, the malaria parasites lack pyrimidine salvage enzymes and depend exclusively on the *de novo* pathway as source of pyrimidines for their survival [5, 33]. *De novo* synthesis from carbamoyl phosphate and aspartic acid follows basically the same steps found in the human host and in other eukaryotes: orotic acid is formed by dihydroorotase (DHOase) and DHODH. The orotic acid is so turned into orotidine 5′-monophosphate (OMP) by addition to 5′-phospo-D-ribosyl-α-1-pyrophosphate, a step carried out by orotate phosphoribosyltransferase (OPRT). OMP is subsequently decarboxylated to uridine 5′-monophosphate (UMP), the precursor of all other pyrimidine nucleotides and deoxynucleotides needed for nucleic acid synthesis [159]. Excepting for *Pf*DHODH, which is discussed in the ETC topic, the enzymes involved in *de novo* pyrimidine biosynthesis pathway that could potentially be exploited for the discovery of novel antimalarials as discussed below.

7.1. Carbamoyl phosphate synthetase II

Carbamoyl phosphate synthetase II (CPSII) is responsible for the first step of the *de novo* pyrimidine biosynthesis, catalyzing the formation of carbamoyl phosphate in the cytosol from bicarbonate, glutamine, and ATP [160]. Differently from the human CPSII, CPSII from *P. falciparum* (*Pf*CPSII) is a monofunctional protein [155]. *Pf*CPSII also differs from its mammalian homologue by the presence of two inserted sequences, located between junctions of the glutamine aminotransferase and synthetase domains [161]. Although the absence of structural information and activity inhibitors, the druggable potential of this enzyme has already been demonstrated by the potent growth inhibitory effect of a synthetic ribozyme with specificity for the *Pf*CPSII gene over *P. falciparum* cultures [162]. The same synthetic ribozyme has shown no toxicity to mammalian cells. Other mini ribozymes were further redesigned to improve cleavage activities and metabolic stabilities [163]. These results suggest that the discover of compounds capable to inhibit *Pf*CPSII in a specific way might be promising antimalarial candidates, since ribozyme approaches have a significant more challenging application due to target accessibility, stability, specificity, and delivery efficiency [164].

7.2. Aspartate transcarbamoylase (ATC)

Aspartate transcarbamoylase (ATC, EC 2.1.3.2) catalyzes the condensation of aspartate and carbamoyl phosphate to form N-carbamoyl-L-aspartate and inorganic phosphate. Previous

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studies with human tumor tissues showed significantly elevated levels of ATC nearly in all samples [165]. In *P. falciparum,* ATC is also present as monofunctional protein, unlike its human homologue. Although a number of publications suggest ATC from *P. falciparum* to be a promising drug target [166–168], it has not been fully characterized and no inhibitors have yet been reported. Recently reported crystal structure of the truncated *Pf*ATC revealed high level of sequence conservation among homologous enzymes from other organisms, especially in the active site area [169].

7.3. Dihydroorotase

Similarly to CPSII, *P. falciparum* dihydroorotase (*Pf*DHOase) is a monofunctional protein and thus differs from the mammalian host, in which the 36.7 kDa enzyme is located on the central part of the 240 kDa CAD multifunctional protein [170]. This enzyme catalyzes the reversible cyclization of N-carbamoyl-L-aspartate (CA-asp) to L-dihydroorotate (L-DHO) [159]. Orotate and a series of 5-substituted derivatives were found to inhibit competitively the purified enzyme from *P. falciparum* culture. In mice infected with *P. berghei*, 5-fluoro orotate and 5-amino orotate at a dose of 25 μg/g body weight eliminated parasitemia after a 4-day treatment, an effect comparable to that of the same dose of chloroquine. The infected mice treated with 5-fluoro orotate at a lower dose of 2.5 μg/g had a 95% reduction in parasitemia [171]. The moderate inhibition of *Pf*DHOase by L-6-thiodihydroorotate (TDHO) in cultured parasites induced major accumulation of CP-asp and growth arrest, similar to atovaquone [172]. The analysis of physical, kinetic, and inhibitory properties of the recombinant *Pf*DHOase performed by Krungkrai et al. suggests that specific inhibitors may limit the pyrimidine nucleotide pool in the parasite, but have no significant adverse effect to human host [173]. Although the low amount of information about *Pf*DHOase does not allow to confirm it as a good candidate to antimalarial development, the report of its crystal structure and biochemical characterization could clarify whether this enzyme is essential or not to the parasite's survivability.

7.4. Orotate phosphoribosyl transferase and orotidine 5′-monophosphate decarboxylase

The last two steps of the pyrimidine biosynthesis in *P. falciparum* are catalyzed by a heteromeric complex that consists of two homodimers of *Pf*OPRT and *Pf*OPDC encoded by two separate genes [174, 175].

The enzyme orotate phosphoribosyl transferase (OPRT) catalyzes the formation of orotidine 5′-monophosphate (OMP) from α-D-phosphoribosyl pyrophosphate (PRPP) and orotate, the fifth step of the pyrimidine biosynthesis [155]. The OPRT inhibitors reported so far includes the compound 5′-Fluoroorotate, an alternative substrate for this enzyme that was shown to inhibit the *in vitro* growth of *P. falciparum* at nanomolar range [176, 177] and to clear parasitemia from *P. berghei*-infected mice [171]. This antimalarial activity is related to the inactivation of malarial thymidylate synthase by 5′-fluoro-2′-deoxy-UMP metabolite through covalent binding to methylene tetrahydrofolate at the active site. The compound pyrazofurin has also been described as a moderate inhibitor of *P. falciparum* OPRT (*Pf*OPRT), inhibiting its activity at micromolar range by blocking the maturation of trophozoites to

schizonts [176, 178]. Interestingly, pyrazofurin does not affect the OPRT activity in mammalian cells [179].

A recent study of the transition state analogues of *Pf*OPRT also showed that despite the tight binding *in vitro*, the synthetized compounds failed to inhibit the parasite culture growth *in vivo* [180–182]. No growth inhibition was observed at high compound concentrations up to 100 μM, suggesting poor compound accessibility *in vivo*.

Recently, the crystal structure of *Pf*OPRT has been reported, which shows a homodimeric assembly, where each of two active sites include amino acids from both chains [183]. Despite the high level of homology with human OPRT, the active site of *Pf*OPRT has few amino acids that differ from *Hs*OPRT. The authors suggest that these differences might lead to the design of selective substrate-like inhibitors in the future.

Orotidine 5′-monophosphate decarboxylase (OPDC) catalyzes the final step of *de novo* pyrimidine biosynthesis pathway, the decarboxylation of orotidine 5′-monophosphate (OMP) to uridine 5′-monophosphate (UMP), with no need for the presence of a cofactor or metal ion [184]. Many inhibitors of plasmodial OPDC have been described so far, being the most promising inhibitor, the nucleotide 5′-monophosphate analogue xanthosine 5′-monophosphate (XMP) [185]. XMP acts as a competitive inhibitor with tighter binding than OMP. The *P. falciparum* OPDC inhibition by XMP is highly selective, having a 150-fold preference for the malarial enzyme compared to human OPDC. Other inhibitors include the 6-iodouridine 5′-monophosphate (6-iodo-UMP) [186], 6-azidouridine 5′-monophosphate (6-N3-UMP) [187], barbiturate 5′-monophosphate (BMP) [185], 6-N-methylamino uridine [187], and 6-N,N-dimethylamino uridine [187]. Although a considerable number of *Pf*ODC inhibitors have been described and a crystal structure of *Pf*OPDC is available [188], a deeper investigation is necessary to clarify *Pf*OPDC as validated drug target.

8. Protein interference assay (PIA) as drug validation tool

We have recently proposed a novel promising drug-target validation approach that relies on common feature of all biological systems—oligomerization [22]. Oligomerization is a self-assembly of two or more copies of one protein molecule (or different molecules) into one object. Recent analysis shows that majority (60%) of non-redundant protein structures available in the Protein Data Bank (PDB) represent dimerization or higher oligomerization order (Hashimoto *et al.*, 2011). In many cases, the biological activity of a protein complex is dependent on correct oligomeric order. Oligomerization may be required for a number of reasons, including the correct active site or cofactor binding site assembly on the oligomeric interface or allosteric regulation. Examples where dimerization is crucial for the formation of active sites on the oligomeric interface include previously mentioned aspartate aminotransferase (AspAT) [22], aspartate transcarbamoylase (ATC), and orotate phosphoribosyl transferase (*Pf*OPRT) [183] from *P. falciparum*. In addition, the physiological assembly of *Pf*OPRT/*Pf*OPDC heterotetramer was shown to be more effective compared to the

http://dx.doi.org/10.5772/65659

monofunctional enzymes [189]. A number of recent publications also suggest that the protein oligomerization to be a key driving force in evolution [190–194].

Another important aspect of oligomerization is remarkable selectivity and binding affinity. Large surface area of the intraoligomeric interfaces and evolutional diversity allow oligomeric partners selectively bind to each other with no cross-reactivity in the system. In majority of cases, purification of oligomeric proteins from both native and recombinant sources can be performed without any foreign protein incorporations in the assembly. Unlike the active sites and cofactor binding sites where evolutionary constraints restrict the sequence diversity to retain the function, oligomeric interfaces are significantly less conserved among homologous proteins [195, 196]. Thus, small molecule compounds reacting with the conserved active site of target enzyme of the parasite will likely interact with the host's homologous enzyme.

Direct interference with protein self-assembly would provide an opportunity for a highly selective modulation of protein activity or function both *in vitro* and *in vivo*.

9. Making (breaking) bad proteins

The recently proposed protein interference assay (PIA) [22] involves the utilization of structural knowledge (data) and mutagenic modification of one (or more) partner proteins in the assembly. These modifications may affect the binding site for a cofactor, catalytic activity, or disrupt the oligomeric interface of the target protein. Thus, recombinant and, most importantly, controlled co-expression of both wild type and its inactive (hyperactive) mutant would allow the formation of the complex with modified activity *in vitro*.

Previously mentioned homodimeric *Pf*OPRT, as part of the *Pf*OPRT/*Pf*ORDC heterotetramer, could also be a subject to PIA. The active sites of *Pf*OPRT were reported to contain the amino acids from both subunits, suggesting that introduction of the active site mutants with modified activity *in vivo* would also affect the native *Pf*OPRT. This assay would potentially bypass previously observed difficulties with poor inhibitor accessibility and aid in validation of the enzyme as antimalarial drug target.

Despite the obvious limitation of PIA approach to oligomeric proteins, this assay would still allow partial assessment of the system of interest, as many of the studied pathways are likely to involve at least one oligomeric assembly. We suggest that PIA would also allow re-evaluation of the previously studied promising targets where conventional validation approaches have failed.

10. Conclusion

In order to assess a gene's product role, one must possess a set of tools, such as genetic manipulations (e.g. knockout, silencing etc.), to modulate the target function *in vivo*. Sufficient specificity (with little or no cross-reactivity) is essential for correct interpretation of the data.

Although genetic manipulations have been proven to be highly effective in model and fully defined systems, less studied and complex systems remain highly challenging. In many pathogenic systems, including human malaria, conventional genetic manipulation techniques or small molecule inhibitor approaches do not always provide the desired efficacy [22]. In a number of human pathogens, multiple life cycle stages in different hosts and vectors make both *in vitro* and *in vivo* target characterization challenging to approach. A number of classic techniques such as silencing RNA [197, 198] have already been reported to be non-effective in certain cases [199–202].

In addition, the use of small molecule inhibitor approaches *in vivo* is associated with high costs and is often limited due to the variety of host-specific reasons that are difficult to predict, such as rapid metabolism, poor membrane transport, or localization. For example, while a number of compounds were reported to inhibit *Pf*OPRT activity *in vitro* as well as clear parasitemia in *P. berghei*-infected mice, *in vivo* trials with *P. falciparum* have failed [180]. Thus, potential drug targets may remain unexplored due to the inability to use the existing validation tool set.

Insufficient amount of effective target validation tools significantly limits the understanding of human pathogenic systems and hinders the rate of novel drug development. A constant supply of robust and effective techniques is needed in order to successfully dissect yet unexplored parasitic pathways, provide the basis for rational drug design, and counter-balance the ability of many human pathogens to rapidly develop drug resistance. We believe that protein interference assay (PIA) will enrich the currently available research toolset.

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Chapter 13

Identifying Antimalarial Drug Targets by Cellular Network Analysis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65432

Abstract

Malaria is one of the most deadly parasitic infectious diseases and identifying novel drug targets is mandatory for the development of new drugs. To find drug targets, metabolic and signaling networks have been constructed. These networks have been investigated by graph theoretical methods. Furthermore, mechanistic models have been set up based on stoichiometric equations. At equilibrium, production and consumption of internal metabolites need to be balanced leading to a large set of flux equations, and this can be used for metabolic flux simulations to identify drug targets. Analysis of flux variability and knockout simulations were applied to detect potential drug targets whose absence reduces the predicted biomass production and hence viability of the parasite in the host cell. Furthermore, not only the parasite was studied, but also the interaction between the host and the parasite, and, based on experimental expression data, stage-specific metabolic models of the parasite were developed, particularly during the red-blood cell stage. In this chapter, these various network-based approaches for drug target prediction will be explained and summarized.

Keywords: network-based analysis, drug targets, flux balance analysis, malaria

1. Introduction

Network-based analysis has become an important tool in biomedical research. It facilitates the investigation and understanding of a system as a whole, not only its single components. For this, first the networks need to be constructed and then investigated employing different analysis or modeling techniques. According to the applied methodological approaches to analyze these networks, one may distinguish cellular network models for signal transduction, gene regulation and metabolism. The network constructions based on information are compiled from databases and are assembled in an automated way often followed by manual refinement. Network-based models have been applied to study the cellular mechanisms of a large variety of diseases elucidating, for example, tumor growth, malfunctioning

of the differentiation of immune cells, or identifying drug targets of invasive pathogens [1, 2]. To find drug targets for the treatment of malaria, metabolic and signaling networks have been constructed and intensively investigated. This chapter will introduce the reader into the basic principles of constructing and applying such cellular networks. It then leads through the application of these systems biology approaches to predict drug targets followed by a small section exemplarily showing an experimental validation for these predictions.

2. Construction of cellular networks

Proteins are involved in all cellular functions. These cellular processes can be put up as cellular networks, which describe associations among these proteins and other cellular compounds such as metabolites and nucleic acids. These cellular networks can conceptually be divided into three distinct parts: the cell signaling, the transcriptional regulatory network, and the metabolic network. The best observed and modeled network is the metabolic network while the complex system of signal transduction is rather captured statistically investigating the experimental information about proteins and their expressed genes of network models basing on protein-protein interactions [3]. The transcriptional regulatory network links transcriptional regulators to their target genes [4]. The simplest form of a network is a network represented by an undirected graph $G = (V, E)$ consisting of nodes V and edges (connections, links) E between these nodes. Each node $i \in V$ represents a unique cellular entity such as enzymes, genes, and proteins, while each edge $(i, j) \in E$ represents an observed interaction between two nodes i and j. A metabolic network model can be constructed as a bipartite graph consisting of two disjoint sets of nodes (reaction and metabolite nodes, see **Figure 1**) [5]. The direction of edges in the metabolic networks is given by the flux from the substrate to the product of a biochemical reaction. An edge indicates that a metabolite is either a substrate or a product of a reaction. The distinction between substrates and products of a reaction is only possible if the graph is directed, that is, if the set of edges E consists of ordered pairs of vertices. This distinction is often useful when modeling metabolic fluxes but may be neglected in simpler models [6]. As a bipartite graph, the metabolic network can be represented as an adjacency matrix of $m \times n$ dimensions, where m is the number of metabolites and n is the number of reactions. More specific models of metabolic networks concerning the stoichiometry can also be represented as an adjacency matrix using stoichiometric coefficients of chemical reactions as weights for the edges between metabolites and reactions. As shown in **Figure 1**, our small example network consists of three reactions (R1, R2, and R3) and six metabolites (A, B, C, D, E, and F):

R1:	A	⇔	B
R2:	2 B + C	→	E + F
R3:	2 E	→	B + D

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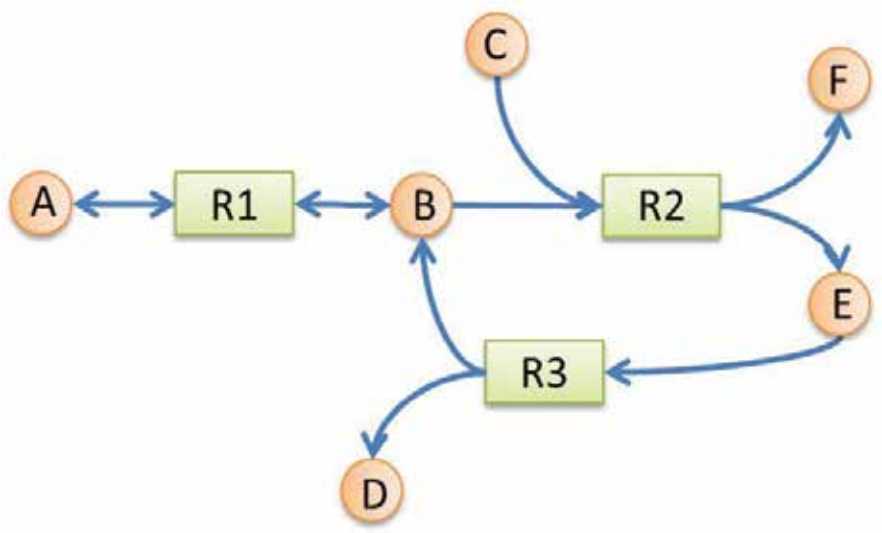

Figure 1. Graphical view of a metabolic network model as a bipartite graph consisting of two disjoint sets of nodes (reactions and metabolites). This network consists of three reactions (R1, R2 and R3) and six metabolites (A, B, C, D, E, F). R1 is a reversible reaction, the other reactions are irreversible.

The stoichiometric matrix or the adjacency matrix containing stoichiometric coefficients of each reaction equation is

$$s = \begin{bmatrix} -1 & 0 & 0 \\ 1 & -2 & 1 \\ 0 & -1 & 0 \\ 0 & 0 & 1 \\ 0 & 1 & -2 \end{bmatrix}$$

where the rows correspond to metabolites A, B, C, D, E, and F, and the columns correspond to reactions R1, R2, and R3, respectively. R1 is a reversible reaction. Metabolic networks for *Plasmodium spp* can be constructed using the databases PlasmoCyc [7], Malaria Parasite Metabolic Pathways (MPMP) [8], The Kyoto Encyclopedia of Genes and Genomes (KEGG), http://www.genome.jp/kegg/, and from models in the literature [9]. Unspecific compounds such as water, ATP, ADP, etc., may be discarded for these rather general models but need to be considered for more detailed models when, for example, employing flux balance analysis (see below). Cellular networks can be analyzed mechanistically or statistically by their topological features. In the following, we explain briefly some of these topological features.

3. Topological features for statistical analyses of cellular networks

Several computational techniques have been developed to identify essential genes and drug targets *in silico* for a therapy against malaria. To construct an undirected graph for metabolism,

the network representation of a reaction-pair network can be used instead of a bipartite graph. In this representation, enzymes are linked if there is at least one metabolite, which is produced by one of the enzymes and which serves as a substrate for the other. For these simple networks, the network topology can be described by characteristic properties. Similarly, protein interaction networks can be analyzed to get specific characteristics for signal transduction [3, 10, 11]. These characteristics either hint directly to essential genes (serving as drug targets) or can be used when comparing the full network with a network in which one of the nodes (enzymes or signaling proteins) is targeted by a drug.

3.1. Diameter and density of a network

The diameter of a network is the largest distance of all shortest paths between two nodes (reactions, signaling molecules) in the network. The density of a network is the ratio of the edges (links, connections) between two reactions divided by all possible edges of all reactions. These two properties can be used to determine the robustness of a network. In recent studies, a reaction was said to be essential if the mutated or targeted network showed a larger diameter after removing the reaction [12, 13].

3.2. Scale-freeness of networks

Networks can be distinguished by their degree distributions where the degree of a node $v \in V$ is defined as the number of edges between v and its adjacent nodes. Many degree distributions of naturally occurring networks follow power laws [6] $P(k) \sim k^{-\gamma}$ where $\gamma > 0$ is a constant depending on the network and is usually in the range of 2–3. P is the probability to draw a node with degree k. Networks with a power law distribution are also called *scale-free* networks [6, 14]. Basically, these scale-free networks consist of few highly connected vertices, so-called *hubs*, and many less connected vertices [15]. Most real-world networks including metabolic networks are approximately scale-free networks [6]. **Figure 2** shows the degree distribution of the metabolic network of *Plasmodium falciparum*, which is fitted by a power-law distribution. Scale-free networks generally have a small diameter [16], as in particular the highly connected nodes connect nodes within only a few links. Additionally, these networks are highly connected [17]. The benefit of such a highly connected and scale-free architecture is its robustness against single "attacks," that is, a failure of a single node in the system, as it is statistically more probable that vertices with lower degrees are hit from which the general structure of the network is not affected. The scale-free topology provides robustness to the network with increases flexibility to random perturbations where the loss of individual nodes usually has no effect on the overall network topology. Nevertheless, such a network is susceptible to targeted attacks at highly connected critical hubs [18], and mutations affecting hubs are more likely to cause a defect [17].

3.3. Clustering coefficient

The clustering coefficient is used to estimate the local density of links (edges) in the network. It describes the connectedness among neighbors and helps to estimate the probability of local alternative paths of signaling or metabolic fluxes (e.g., after targeting). The clustering coefficient of a node v is defined as the ratio of the number of connecting edges among all neighbors

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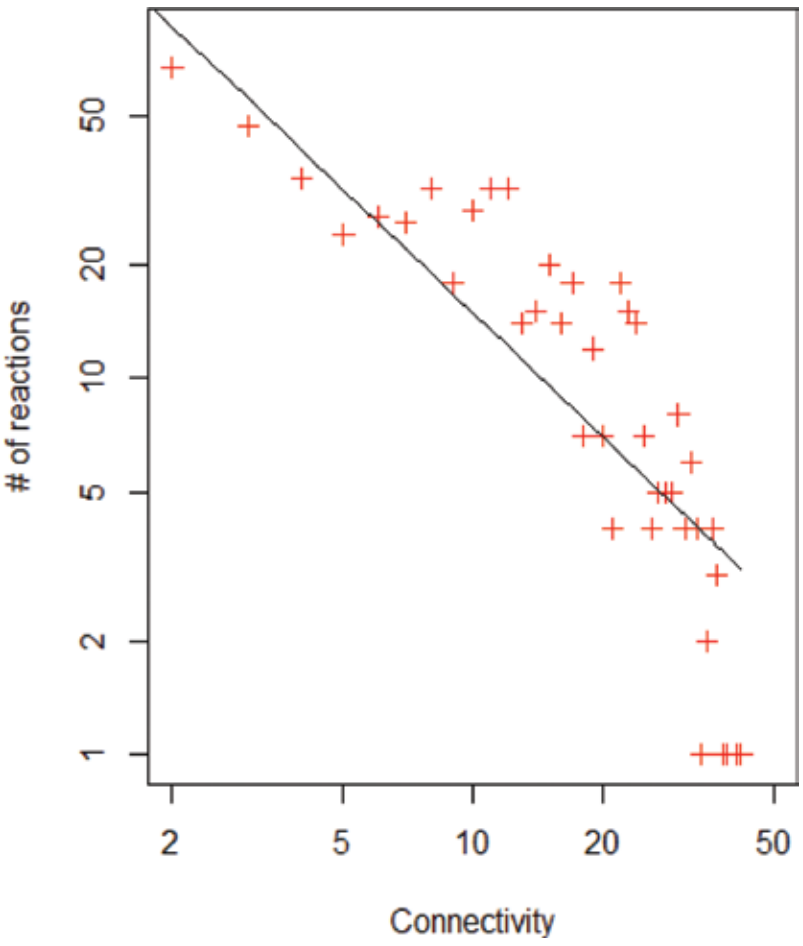

Figure 2. Degree distribution of the metabolic network of *P. falciparum* using the (most suitable) network of [13].

of v and the total number of edges among them that could be possible. This means, if all neighbors are connected among themselves, the clustering coefficient becomes one, if none of the neighbors is connected with any other neighbor, it is zero [6, 19, 20]. In **Figure 3**, the observed reaction in dark has three neighbors and two edges among its neighbors. Having three neighbors, there are six possible connections among neighbors. Thus, the clustering coefficient of the observed reaction in this example can be computed as $\frac{2}{6} = \frac{1}{3}$.

3.4. Centrality

Descriptors for *node centrality* are quite powerful for describing the potential of essentiality of a node. They describe not only the impact of the node to its direct vicinity but also the contribution of a node to the global structure of the network. The simplest of all centrality measures is the connectivity, or degree k, which is just the number of links connecting the node with other nodes. In a cellular network, the degree is commonly used to describe an important node as it

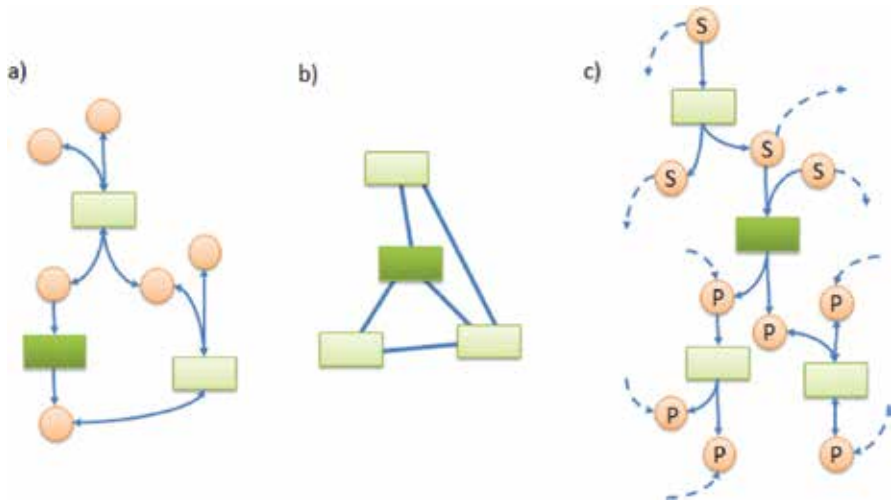

Figure 3. Illustration of the concepts of the topology features. Circles represent metabolites, rectangles reactions, arrows directions of the metabolic flux, lines represent links between two neighboring reactions and dark rectangles represent the investigated reactions. (a) The observed reaction is a chokepoint because it is the only reaction consuming the upstream metabolite. (b) The metabolic network in a reaction-pair representation for computing the clustering coefficient. The observed reaction has three neighbors (degree of 3) and there are two links among these neighbors. Therefore, the clustering coefficient for this observed reaction is 1/3. (c) Graphical illustration of the way to compute producibility of the observed reaction from its substrates (S) to its products (P). Possible alternative pathways to consume substrates S for producing products P are represented by dashed arrows. The percentage of the products that can be produced from the substrates is the producibility of the observed reaction.

is known that often essential genes are nodes in the network with a high degree (so-called hubs). Another commonly used centrality measure is betweenness centrality. Betweenness centrality is the frequency of a node to be part of the shortest paths connecting all pairs of nodes in the network [21].

3.5. Choke points and load points

In metabolic networks, Samal *et al*. found out that most reactions identified as essential are involved in the consumption or production of metabolites with low connectivity [22]. This is because these nodes are more likely to be the limiting factor for consuming or producing these metabolites. In the extreme case, an enzyme is the only enzyme, which consumes or produces a certain compound. Blocking such a reaction may cause severe effects to the cell as, for example, it may cause an assembly of toxic compounds, which cannot be degraded anymore or a lack of substrates for important processes further downstream of the enzyme. Hence, a choke point reaction was defined as a reaction that uniquely consumes or produces a certain metabolite in the metabolic network [23, 24]. This concept has been successfully applied to identify drug targets for *Plasmodium spp.* [24, 25]. Load scores are defined as hot spots in the metabolic network (enzymes or metabolites) based on the ratio of the number of shortest paths (connecting any two enzymes or metabolites in the whole network) passing through a metabolite or enzyme and the number of nearest neighbor links [23].

3.6. Producibility (by deviations)

A reaction is determined to be potentially essential when basically the mutated network cannot yield the products of the reaction from upstream substrates of the reaction using other pathways linking the substrates to the products (see **Figure 3**). The percentage of the products that can be produced from the substrates, the so-called "producibility," can be used to examine the essentiality of the observed reaction [13].

3.7. Applying these topology-based methods to predict drug targets for *Plasmodium* spp.

The concept of choke points and load points was successfully applied to estimate the essentiality of an enzyme in *Plasmodium* [23, 24, 26]. Yeh *et al*. initially applied a chokepoint analysis for *P. falciparum.* Strikingly, they found that 87% of known drug targets with biological evidence are chokepoints according to their analysis [24]. In line, they identified three targets of clinically proven malaria drugs, dihydrofolate reductase, dihydropteroate synthase, and 1-deoxy-D-xylulose 5-phosphate reductoisomerase as chokepoints. Rahman and Schomburg performed a chokepoint and load score analysis for several other organisms [23, 26]. In Fatumo *et al*., we performed a chokepoint analysis together with our developed producibility concept to obtain a more reliable list of potential drug targets in *P. falciparum*. For example, we identified deoxyhypusine synthase involved in spermidine metabolism, which is a known drug target in *P. falciparum*, *Anopheles stephensi*, and *Trypanosoma evansi* [27]. This enzyme was detected by intersecting the predicted targets from a chokepoint and a producibility analysis [26].

Protein-protein interactions were inferred by a high-throughput method (yeast-2-hybrid) and assembled for a signaling network of *P. falciparum.* This has been performed for the first time by Suthram in 2005 identifying conserved proteins, pathways, and interactions [28]. The network was then analyzed by using a network alignment approach comparing the networks across organisms, by using various graph theoretical measures and an *in silico* knock-out strategy to identify potential drug targets [11, 12, 28, 29]. With this, conserved pathways and proteins between organisms were identified hinting for essentiality. The study showed that a few interactions were conserved among the analyzed organisms, demonstrating that the protein interaction network of *Plasmodium* is distinctively different from the others. Interestingly, a conserved protein complex was found in calmodulin-mediated endocytosis. Indeed, inhibition of calmodulin resulted in attenuated growth [30] and reduced chloroquine extrusion in malarial parasites diminishing drug resistance to chloroquine [31]. Additionally, endocytosis was found to be related to these mechanisms [32]. Thus, the proximity of calmodulin to the formation of endocytic vacuoles in *Plasmodium* provides an interesting link to discover strategies coping drug resistance mechanisms of *Plasmodium* [28].

Recently, Bhattacharyya and Chakrabarti analyzed a large-scale protein-protein interaction network of *Plasmodium* and identified potential drug targets using various graph theoretical measures such as centrality measures. They also used an *in silico* knock-out strategy to study the perturbation due to a loss of a protein in the network [12]. With this, approximately 270 proteins of *P. falciparum* were identified as potential drug targets including proteins, which play crucial roles in intra-pathogen network integrity, stage specificity but also interact with

various human proteins involved in multiple metabolic pathways within the host cell. Most of the housekeeping proteins were found to be potential targets [12].

Interactions between the human host and the parasite have been intensively studied [11–13, 33]. The comparison of several reconstructed network models has been performed to find the best suitable reconstruction for detecting drug targets *in silico*. This was performed on a metabolic network reconstruction based on automatically inferred enzymes and compared with a reconstructed model that based only on enzymes whose coding genes were known. These networks were analyzed with criteria for defining essential enzymes including chokepoints, betweenness centrality, connectivity, and the diameter of the networks. Comparing the modeling results with a comprehensive list of known drug targets for *P. falciparum* showed that the most suitable network model was constructed using only enzymes from the parasite alone, which coding genes were known [13].

Chen *et al*. developed a network-based approach to predict malaria-associated genes by a random walk algorithm [33]. They first constructed separate gene networks of the human genome and of the parasite genome and then connected them with known host-pathogen protein interactions. Known malaria target genes were used as the seeds (a set of nodes at which the search started) in a random walk algorithm to prioritize genes. The random walk algorithm then iteratively explored the global structure of the network starting at a set of nodes (seeds) to estimate the probability of a node being reached from the seeds. These probability scores can be viewed as the influential impact over the network imposed by the set of seed nodes. Finally, all the genes were ranked according to their probability scores. Manually examining the top 50 predicted human genes, interesting proteins such as TLR4 and P53 were found to be associated with malaria [33].

4. *In silico* modeling using flux balance analysis to identify drug targets

Flux balance analysis (FBA) is a computational approach to estimate the quantitative flux of metabolites through a mechanistic model of metabolism. Thereby, it is possible to predict the growth rate of an organism or the rate of production of an important metabolite [9, 34–36]. Biochemical stoichiometric equations are used to assemble a set of constraints to limit the feasible search space. The idea is that, at equilibrium, production and consumption of internal metabolites are balanced. This leads to a large set of equations in which the net production flux equals the net consumption flux for each internal metabolite. Additionally, allowable fluxes of any reaction are bounded at plausible maximum and minimum fluxes. Bounds may also be taken from the literature. These balances and bounds define the space of allowable flux distributions of a system, that is, the allowed combinations of fluxes for each reaction. To get a phenotype or modeling prediction from these constraints, an optimization criterion is put up. For example, in the case of predicting growth, the objective is to optimize biomass production which is the rate at which metabolic compounds are converted into the physiological portions of biomass constituents most importantly of nucleic acids, amino acids and lipids. Together with the constraints, this is mathematically formulated as a system of linear equations which is solved using linear programming based programs. Flux variability and knockout simulations

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are analyzed to detect potential drug targets whose absence reduces the biomass production and hence viability of the parasite in the host cell. By simulating a reconstructed metabolic network of an organism of interest, first a "wildtype" model is investigated and the growth rate of the wildtype under specific bounds (or conditions) obtained. Performing a single gene (or reaction) knockout/deletion under the same condition by limiting its corresponding fluxes to zero (knockout simulation), the fluxes are calculated simulating an organism effected to a drug (targeting the deleted enzyme) and the growth rate is compared to the wildtype. A knocked out gene (or reaction) is predicted to be essential under the given condition if the mutant model yields a much lower growth rate compared to the wildtype. Flux balance analysis is a widely used and well-established technique to assess the essentiality of genes and hence potential drug targets [9, 34–36]. The beauty of this approach is that it does not depend on specific enzymatic parameters for each enzyme like their Michaelis Menten constants, etc., but are rather basing on simple stoichiometric equations. To some extent, the only experimental parameters are the boundary conditions. The drawback is that often several solutions can come out which are mathematically equally good, but physiologically very different leading to follow-up analyses of each of these solutions. Nevertheless, the approach was used for several genome-scale metabolic network constructions, followed by flux simulations of the inner metabolites of *Plasmodium* spp. to identify drug targets. It also enables to embed the metabolism of *Plasmodium* spp. into the metabolism of its environment, for example, human red blood cells [9]. Furthermore, experimental data on a systems view can be embedded using microarray or sequencing based gene expression data and with this, stage-specific metabolic models of the parasite were developed, particularly during the red-blood cell stage [9]. To better understand flux balance analysis and its potential, we will give a brief introduction into the mathematical secrets of it in the next section (which can be skipped without losing the track to understand the subsequent sections).

4.1. Flux balance analysis formulation

Let s_{ij} be the stoichiometric coefficient of metabolite i in reaction j, which specifies the number of metabolites produced or consumed by reaction j. $s_{ij} > 0$ indicates that reaction j produces metabolite i, while $s_{ij} < 0$ indicates that reaction j consumes metabolite i. $s_{ij} = 0$ means that metabolite i does not participate in reaction j. For example, considering a reaction $A + 2B \rightarrow C$, the stoichiometric coefficients of A, B, and C are −1, −2, and 1, respectively. The stoichiometric coefficients s_{ij} can be combined into the so-called *stoichiometric matrix* $S = (s_{ij})$. A rate of concentration change of a metabolite can be formulated by the set of system equations:

$$\frac{dx_i}{dt} = \sum_j s_{ij} v_j \tag{1}$$

where x_i is the concentration of metabolite i, s_{ij} is the stoichiometric coefficient, and v_j is the consumption/production rate of reaction j. Based on the assumption of mass conservation at steady state in the cell, internal metabolite concentrations are constant over time. Therefore, the concentration change of each internal metabolite i is zero, which means $\frac{dx_i}{dt} = 0$. With this assumption, equation (1) can be formulated as

$$\sum_j S_{ij} v_j = Sv = 0 \tag{2}$$

where S is the $m \times n$ stoichiometric matrix of m metabolites and n reactions in the network. The vector v represents all reaction rates (also called metabolic fluxes) in the metabolic network. The ranges of individual metabolic fluxes are constrained by $\alpha_j \leq v_j \leq \beta_j$ where α_j and β_j are the minimal and maximal fluxes of reaction j, respectively. These inequality constraints allow reversibility. If a reaction is reversible, the flux of the reaction v_j can either be negative or positive. A positive flux indicates the forward direction while a negative flux indicates a backward direction. If we want to block a reaction (knockout simulation), we can constrain the flux of this reaction to be equal to zero ($v_j = 0$). In addition, the benefit of these inequality constraints is to simulate metabolic capabilities under certain conditions such as a glucose minimal medium condition, which we can model by constraining the flux of the glucose uptake rate in a specific range of values and set the uptake rates of all other carbon source to zero. Finally, the set or subspace of vector v that satisfies all constrains and the ranges of individual metabolic fluxes is a set of feasible fluxes covering all feasible capabilities of the metabolic network under the given specific condition. Using an optimization criterion, such as to optimize the biomass of the cell yields then only one or a few out of these solutions. The biomass production rate can be defined by a reaction or several reactions that produce the metabolic building blocks of a cell (e.g., amino acids and nucleotides) or macromolecules that form the biomass in a physiological composition. The physiological biomass composition of a given organism comprises the relative amounts of the important molecules and can be found in the literature [9, 36]. The flux of the biomass production is associated with the specific growth rate of an organism. Finally, the obtained growth rate of the mutant (with a reaction knocked out) is compared to the growth rate of the wildtype to predict a gene or an enzyme to be essential. This section was taken from Ref. [37].

4.1.1. Applying FBA to predict drug targets

FBA has been widely used to predict essential genes of the human malaria parasite *P. falciparum* [9, 36, 38]. A metabolic network reconstruction of *P. falciparum* was developed with 1001 reactions and 616 metabolites [36]. The model allowed predicting the phenotype (growth) of experimental gene knockouts. Validating the predictions with drug inhibition assays yielded approximately 90% accuracy. Several modifications on the linear programming implementation were studied to make the static FBA model more realistic. For example, gene expression profiles of the malaria parasite were integrated into metabolic models [9, 36, 38]. In the study of Plata *et al*. [36], the maximum flux of the associated reactions was constrained by their expression level while Huthmacher *et al*. [9] used a method proposed by Shlomi *et al*. [39]. This method is a modification to flux balance analysis (FBA) by adding binary variables for each reaction. These binary variables act like an on/off switch according to the expression level. The mathematical objective is to maximize the number of non-zero fluxes for the reactions with switched-on-state. Dholakia *et al*. analyzed many available *omics* resources of stage-specific expression and used pathway tools from the BioCyc database to analyze flux distributions with respect to gene expression for identifying drug targets, and in particular in the erythrocytic stage-specific metabolism of the parasite. Based on the FBA approach, Plata *et al*. identified 40 enzymatic drug

targets. All of these enzymes had no or very low sequence similarity to human proteins which made them more attractive as this facilitated designing drugs targeting these enzymes and not human host factors. This set of genes consisted of six genes associated with isoprenoid metabolism, three genes involved in nucleotide metabolism, and the rest of genes related to CoA, shikimate, and folate biosynthesis. In addition, one of predicted essential genes, nicotinate nucleotide adenylyltransferase, was selected to be tested further in an experimental assay. This enzyme has been known for anti-microbial development [40] but not in *Plasmodium* spp. yet. Thus, in Plata et al., the experimental validation was done in *P. falciparum* by inhibiting this enzyme by a small-molecule inhibitor from [41] resulting in blocking host cell escape and reinvasion by arresting the parasites in the trophozoite growth stage [36]. Hence, FBA allowed the construction of stage-specific metabolic networks for different stages of the parasites and gave the opportunity to find drug targets for these stages. Additionally, also host-parasite interactions can be studied using FBA [9]. In the study by Huthmacher et al. [9], a host-parasite network was constructed and the metabolic fluxes for each blood life cycle stage were predicted employing gene expression data of the different stages. Knock-out simulations identified 307 indispensable metabolic reactions for the parasite. Of 57, 35 experimentally validated essential enzymes were recovered. Another set of 16 enzymes were predicted, if additionally assuming that nutrient uptake from the host cell is limited and all reactions catalyzed by the inhibited enzyme are blocked. An interesting modification to flux balance analysis was developed by a two-stage flux balance analysis to identify drug targets by comparing the differences of fluxes between a drug treated and untreated condition [42]. This approach was applied to find drug targets in *Plasmodium*, which is described in more detail in the next section.

4.2. Finding multiple drug targets to treat a drug resistant *Plasmodium* strain

Recently, Phaiphinit et al. reconstructed the metabolic network of *P. falciparum* in the human host red blood cell using flux balance analysis [35]. This model was used to analyze two specific metabolic models: a model for the parasite when having invaded the red blood cell without any treatment and, in turn, the treated situation, when a drug like chloroquine acts by inhibiting the hemozoin formation causing a high production rate of harmful heme. The process of identifying target combinations consisted of two main steps (**Figure 4**):

Step 1—Developing two multi-cellular metabolic models: The model was constructed for the situation of the parasite being inside the red blood cell of the human host. All metabolites of the parasite in exchange with the external environment were taken from the red blood cell. To find potential reactions which could harm *P. falciparum* by getting exposed to severe toxicity, the flux distribution of the multi-cellular metabolic model was calculated for two conditions. The first condition was the untreated situation where the parasite was able to get rid of toxins from hemoglobin degradation after consuming hemoglobin from the red blood cell. The second condition mimicked the treated situation in which the toxins could not be degraded. The difference in flux distributions between the two conditions was assumed to be the effect from the drug which disturbed the parasite.

Step 2—Finding the optimal drug target: The reactions in the parasite which were disturbed from the drug in the treated situation may suit as drug targets for a combined treatment, or if

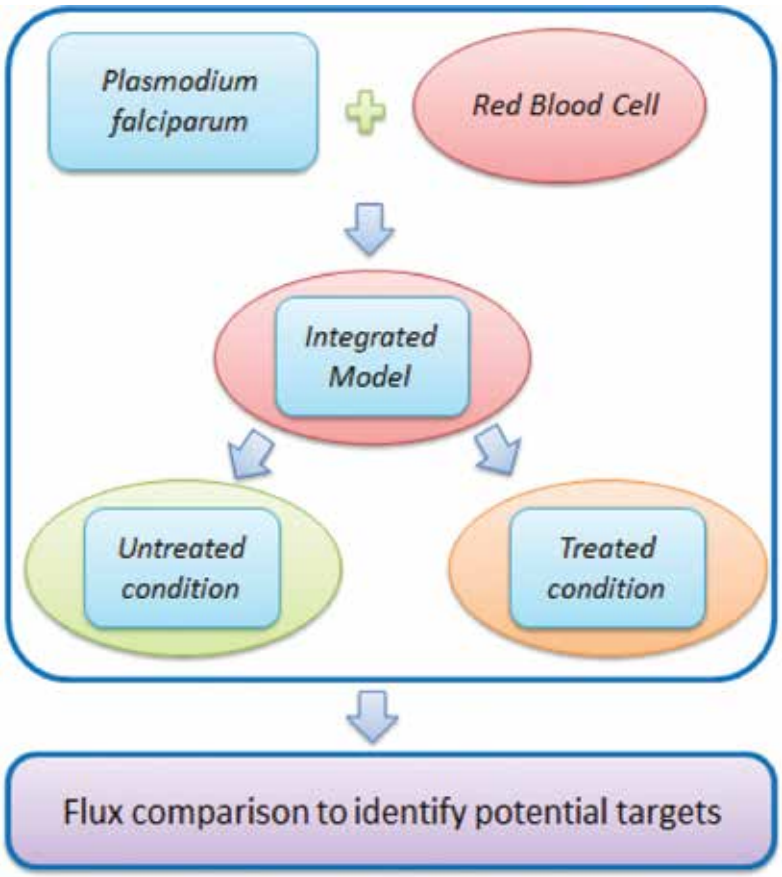

Figure 4. The workflow to identify drug targets by the comparison of treated and untreated conditions. First, *in silico* models of *Plasmodium falciparum* and the human red blood cell are combined as an integrated model. This integrated model is characterized by two specific conditions (treated and untreated). The flux rates of all reactions in both situations are compared to identify a set of potential drug targets.

the parasite gets resistant to the first drug (chloroquine). In particular, reactions for which no flux was predicted in the treated scenario were promising targets because they may have a similar treatment effect when targeted compared to the original drug and may suit as drug targets against strains which are resistant to the first drug.

FBA was used to get the flux distributions for the untreated and the treated conditions. For the untreated condition, the objective was to maximize the production rate of biomass according to Ref. [36], including the Na^+/K^+ ratio based potential at the ATPase, which plays an important role for the homeostasis of red blood cells [43, 44]. In the treated condition, the drug usually inhibits the detoxification process of the parasite harming the parasite due to the toxicity of free heme. Thus, during the treated condition, the (toxic) flux of heme production should be an additional objective to ensure that the toxic flux is not zero when identifying reactions or enzymes to be blocked during the treatment. The flux distributions of both models were then compared to obtain a list of candidate targets by the criteria that the reactions with zero fluxes

in the treated condition but non-zero fluxes in the untreated condition could be potential targets for inhibiting heme detoxification.

With this method, 23 enzymes were identified as candidate targets, which mostly were in pyruvate metabolism and the citrate cycle. The optimal set of multiple targets for blocking the detoxification was a set of a heme ligase, adenosine transporter, myo-inositol 1-phosphate synthase, ferrodoxim reductase-like protein, and the guanine transporter. Purine transporters have been known as the major route of purine into the parasitized red blood cell. In the development of anti-malarial drugs, inhibitors targeting purine transport are of pharmaceutical interest and are investigated. Likewise, adenosine transport and its inhibitor have been studied in infected and uninfected human erythrocytes recently [45]. In summary, this shows an efficient way to identify useful target combinations in the development of novel antimalarial drugs [35].

5. Experimental validation, a case study

Typically, after the computational network analysis, a list of potential drug targets is assembled and needs to be validated experimentally. Exemplarily, in one study of a topological network analysis, 22 potential targets were proposed [26]. Using a refined network comprising also the host enzymes led to a refined set of the five potential drug targets (glutamyl–tRNA (gln) amidotransferase, hydroxyethylthiazole kinase, deoxyribose–phosphate aldolase, pseudouridylate synthase, and deoxyhypusine synthase) [46]. The next step was to find effective inhibitors to block these enzymes. Many reported inhibitors can be collected from databases like the Brenda Enzyme database [47], Drugbank [48], and from companies like Sigma (http://www.sigma.com), or by scanning the literature. In this example, a study was found, in which Jahn and coworkers used 6-diazo-5-oxonorleucine (DON) to be an effective inhibitor of glutamyl-tRNA(Gln) amidotransferase in *Chlamydomonas reinhardtii* [49]. Accordingly, an experimental viability assay (IC_{50} analysis) was performed and showed that DON suits as a valid agent against *P. falciparum* (laboratory strain Dd2) in *Plasmodium* infected blood cultures. Strikingly, this was confirmed by an *in vivo* study using *Plasmodium berghei* infected Swiss albino mice. All treated mice survived whereas all untreated died [45].

6. Conclusions

Even though the number of deaths caused by malaria has diminished considerably, it is still a challenge to treat the effected patients and clear off the pathogen after infection. In particular, there are increasingly more strains getting resistant against common treatments, and hence there is a striking demand to find new targets for therapy.

The computational approaches introduced here show some convincing results. However, it needs to be shown that these predictions are experimentally confirmed and finally make their way from the bench to the bedside.

Various techniques of network-based analyses to identify potential drug targets of *Plasmodium* have been described in more detail including the construction of cellular networks, the

analysis of topological features, as well as *in silico* models based on flux balance analysis. To construct a network, one needs to consider the network types which are suitable to find the targets of interest. Moreover, the consideration of the interactions between host and pathogen makes the network more realistic, but, however, also more complex to obtain drug targets. Analyzing topological features seems to be a comfortable way to retrieve interesting targets; however, the *in silico* models using flux balance may reflect much more detailed relations of the biochemical reactions in a cell. All of the methods described in this chapter provided promising results, some with experimental evidence. It is to be noted that they have been widely used for a large variety of other organisms as well.

Even though all these presented concepts have the very same aim to find a target, their results are quite heterogeneous lists of different predicted drug targets, some of them validated by experimental assays. As a future aspect, a data and method integration needs to be performed leading to a *consistent* set of targets independent from the data it bases on, and, at its best, being consistent with a larger set of experimental data sets and validations.

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Chapter 14

Tackling the Problems Associated with Antimalarial Medicines of Poor Quality

Kamal Hamed and Kirstin Stricker

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64671

Abstract

The use of poor-quality antimalarials has devastating consequences, including increased morbidity, mortality, and drug resistance. Unfortunately, this issue appears to be widespread, especially in parts of Africa and Asia, jeopardizing the progress and investments already made in global malaria control in these regions. In developing countries, inadequate laws and regulatory oversight, along with the lack of human, technical, and financial resources, do not encourage the manufacture and distribution of high-quality medicines. The problem of poor-quality medicines can only be addressed by a multipronged approach that includes tackling poor regulation and ineffective/poorly implemented laws at national and international levels. In addition, pharmaceutical companies must be responsible for ensuring that the quality of antimalarials meets the stringent guidelines established by regulatory authorities, for testing their medicines accordingly and for releasing to market only medicines that pass these requirements. The chapter also discusses how the implementation of strategies such as the WHO Prequalification Program, the African Medicine Registration Harmonization initiative, and the ethical production of medicines by pharmaceutical companies help to ensure that antimalarial therapies marketed in low-income, malaria-endemic countries are quality assured.

Keywords: antimalarial medicines, malaria, quality, Africa, Asia

1. Introduction

Safe, effective, high-quality, and affordable medicines are essential for the achievement of positive, equitable health outcomes [1]. They are necessary for the prevention and treatment of serious public health threats, and for the achievement of the global health goals [2]. However,

there is growing concern regarding the increasing availability and use of poor-quality medicines, particularly in developing countries in Africa, Asia, and South America [3–7].

Poor-quality medicines include those that have been falsified (i.e., deliberately and fraudulently mislabeled with respect to identity and/or source) and substandard medicines (i.e., products resulting from poor manufacturing with no intent to deceive and usually with inadequate or too much active pharmaceutical ingredient) [2].

Falsified medicines have resulted in billions of dollars in illegal annual revenues going to criminals and have caused prolonged, severe illness and deaths. Falsified drugs particularly affect the most disadvantaged people in poor countries [5].

In particular, the use of substandard antimalarial agents has been reported [3, 4, 7–18]. A World Health Organization (WHO) survey of antimalarial medicine quality in six countries from sub-Saharan Africa discovered that nearly 30% of the fully tested samples of medications failed to comply with internationally recognized quality specifications [8]. Similarly, a review reported that approximately one third of antimalarial medication samples from sub-Saharan Africa, as well as from Southeast Asia, failed chemical assay analysis [3].

Factors contributing to poor-quality medicines are numerous [3]. In many developing countries, for example, first-line artemisinin-based combination therapy (ACT) cannot be afforded by many patients [4, 5]. Consequently, patients tend to procure cheaper alternatives that may be falsified or substandard and that may contain subtherapeutic amounts of the active pharmaceutical ingredients (APIs) or only one of the two active ingredients of the ACT [2, 12]. Thus, the use of such poor-quality antimalarial agents leads to increases in morbidity and mortality [19]. In addition, subtherapeutic concentrations of drugs in vivo may be contributing to the selection of resistant parasites [3, 20, 21]. In the face of seemingly ineffective agents, societies are at risk of losing confidence in antimalarial medicines, their doctors and the healthcare system, and thus jeopardizing years of global public health success and investment [2].

In many countries, inadequate laws and regulatory oversight, along with the lack of human, technical, and financial resources, do not encourage the manufacture and distribution of high-quality medicines [2, 22]. The WHO has estimated that nearly a third of countries lack the ability to oversee medicine manufacture, importation, or distribution [23]. With this in mind, the WHO is playing an important role in establishing quality standards for registration and quality control of antimalarials by a prequalification program [24]. Initiatives such as the African Medicine Registration Harmonization (AMRH), in which partners collaborate with the WHO, have also been started in an attempt to improve quality standards of medicines at local and regional levels [25]. In addition, pharmaceutical companies must be responsible for ensuring that the quality of antimalarials meets the stringent guidelines established by regulatory authorities, for testing their medicines accordingly and for releasing to market only medicines that pass these requirements [2, 22].

This chapter will discuss the extent and consequences of poor-quality antimalarial medicines. In addition, the ways in which this issue might be tackled are also discussed, with a focus on the role of pharmaceutical companies, the WHO, and local and regional initiatives.

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2. Defining poor-quality antimalarials

A variety of definitions have been used to classify the different types of poor-quality medicines [3, 26, 27]. The WHO has attempted to develop a consensus on definitions of poor-quality medicines, and has outlined two classes of drugs, namely those that are falsified, and those that are substandard [26].

Falsified medicines have been fraudulently manufactured with fake packaging, and contain little or no active ingredient (and often other potentially harmful substances) [3, 26]. Falsified antimalarial tablets and ampoules containing little or no API are a major problem in some areas [25]. They may be impossible to distinguish from the genuine product and may lead to under-dosing and high levels of treatment failure. In some extreme cases, the falsified antimalarials may contain toxic ingredients [26].

Substandard medicines have been poorly manufactured by a legitimate producer with no intent to deceive, but they usually have inadequate or excessive amounts of active ingredient(s) and/or excipients [3, 26]. The WHO also includes degraded drugs within this class [26]. The degraded drugs were originally of good quality, but become poor quality as a result of unsuitable or extended storage after manufacturing, or through interaction with inadequate excipients [3, 26].

3. The growing problem of poor-quality antimalarials

The problem of poor-quality antimalarial agents, particularly those containing artemisinins, is widespread and varies among countries. There are numerous reports of falsified and substandard antimalarial agents in particular in Africa and Asia [8–18].

One WHO survey evaluated the quality of selected antimalarials in six countries in sub-Saharan Africa (Cameroon, Ethiopia, Ghana, Kenya, Nigeria, and United Republic of Tanzania) [8]. Samples were collected and tested for quality by reliable quality control laboratories according to specifications set up in recognized pharmacopeias. The researchers found that 28.7% of the 267 fully tested samples collected between April and June 2008 failed to comply with prespecified internationally acceptable quality criteria. A similar proportion of ACTs and sulfadoxine-pyrimethamine (SP) were subject to quality defects (29% for ACTs and 28% for SP; **Figure 1**). Prevailing problems associated with ACTs were related to the content of the APIs and the presence of impurities. For SP, it was mainly problems related to dissolution. Interestingly, only 4% of the drugs with the WHO prequalification status failed quality analysis, compared with 60% of drugs not prequalified by the WHO. Therefore, control of the quality of antimalarial medicines throughout the distribution system, according to proper specifications, is an important prerequisite for ensuring optimal treatment outcomes [8].

In Southeast Asia, a similar problem with poor-quality medicines has been reported in a review of published and unpublished data from studies evaluating samples of antimalarials [3]. In seven Southeast Asian countries, 497 (35%) of 1437 samples failed chemical analysis, 423 (46%)

of 919 samples failed packaging analysis, and 450 (36%) of 1260 samples were falsified [3]. The same review also found that, in sub-Saharan Africa (21 countries), 796 (35%) of 2297 samples failed chemical analysis, 28 (36%) of 77 samples failed packaging analysis, and 79 (20%) of 389 samples were falsified [3].

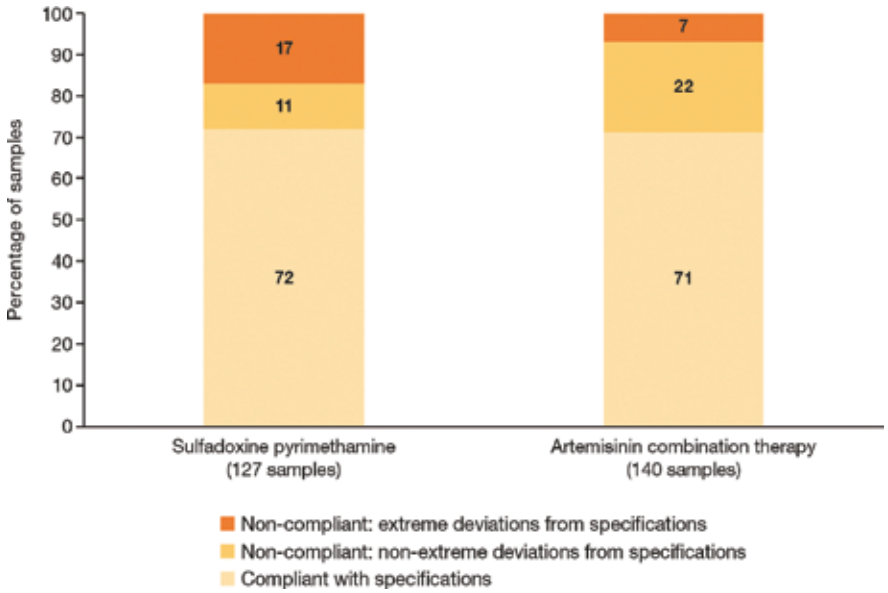

Figure 1. Proportions of compliant, moderately noncompliant and extremely noncompliant samples of artemisinin-based combination therapies (ACTs) and sulfadoxine-pyrimethamine (SP) in sub-Saharan Africa [8]. Extreme deviations were defined as a deviation by at least 20% from the declared content to one or more active ingredients, and/or dissolved percentage of one or more active ingredients less than the pharmacopoeial limit (*Q*) minus 25%.

The World-Wide Antimalarial Resistance Network (WWARN) has developed a comprehensive, open-access, global database, with a linked Antimalarial Quality Surveyor (an online visualization tool) in order to more fully understand the evidence relating to poor-quality antimalarials [15]. A systematic literature search from 1946 to March 2013 identified 251 published antimalarial reports, of which 130 had sufficient information to estimate the frequency of poor-quality antimalarials [15]. Out of 9348 antimalarials sampled, 30.1% (2813) failed chemical or packaging quality tests. Of the 2813 failed samples, 39.3% were classified as falsified, 2.3% as substandard, and 58.3% as poor quality without evidence available to categorize them as either falsified or substandard. Oral artesunate was the medicine most commonly reported as falsified (with 61.9% failing). The survey found that 60.6% (63) of the 104 malaria-endemic countries had no publicly available reports on antimalarial drug quality. Further investigation of the quality of antimalarials is required in the Americas, and in central and southern African regions, as there have been very few studies conducted in these malarious areas [15].

Studies from individual countries have also reported the existence of poor-quality antimalarials. One recent study used mystery shoppers and overt surveys to identify the quality of

antimalarials in Cambodia [12]. Of 291 samples tested, 31.3% did not contain an appropriate amount of the API, i.e., the API content was not within the 85–115% range when measured by high performance liquid chromatography, and the samples were therefore considered to be of poor quality.

A recent study of the quality of artemisinin-based antimalarials from Tanzania's private sector found that, while none of the 1737 antimalarial samples were falsified, 4.1% were outside the 85–115% artemisinin API range [17]. WHO prequalified drugs (25.7% of the total) were more likely to be of higher quality, with only 0.5% WHO prequalified drugs being of poor quality, compared with 5.4% of those not WHO prequalified.

4. Consequences of poor-quality antimalarials

The use of poor-quality antimalarials has serious consequences (see **Table 1**) [3, 19, 28–36].

Increases in morbidity and mortality
Financial loss for patients, healthcare system, pharmaceutical companies
Loss of public confidence in pharmaceutical brand, medicines, pharmacies, and healthcare providers
Increased drug resistance

Table 1. Consequences of poor-quality antimalarials.

4.1. Morbidity and mortality

Exposure to low-quality antimalarials results in poor treatment outcomes, with increased morbidity and mortality. The literature contains several case reports of patients placed at risk or dying as a result of poor-quality antimalarials [28–30]. A study in 39 sub-Saharan African countries estimated that approximately 122,350 (4%) of the deaths in children aged <5 years that occurred in 2013 were associated with poor-quality antimalarial agents [19].

The impact of falsified and substandard medicines is likely to extend beyond such increases in morbidity and mortality. The continued use of poor-quality antimalarials that carry subtherapeutic levels of active ingredient will most likely lead to drug resistance [26, 31–36]. Partial artemisinin-resistant *Plasmodium falciparum* malaria has been reported in several Asian countries in the Greater Mekong Subregion [31–35]. The change in parasite sensitivity is manifest in the form of delayed parasite clearance, and has been associated with mutations in the Kelch 13 (K13) propeller region [37]. Indeed, the Greater Mekong Subregion is a major source from which drug-resistant malaria, including artemisinin-resistant *P. falciparum*, is known to emanate [38]. This occurs for multiple reasons, among which the local prevalence of falsified or substandard artemisinins is an important exacerbatory factor [38]. Based on the new WHO Global Technical Strategy for malaria 2016–2030, countries in the Greater Mekong Subregion have established a Strategy for Malaria Elimination in the Greater Mekong (2015–

2030) in response to the threat of multidrug resistance in this region [36, 39]. The goals of the strategy are to eliminate *P. falciparum* malaria by 2025, and all malaria by 2030, in all countries in the Greater Mekong.

4.2. Resistance

Resistance can be prevented or its outset slowed by the use of combination antimalarials, with different mechanisms of action [26]. Consequently, the standard of care for uncomplicated *P.falciparum* (i.e. move P. from previous page to page 6, line 1 so as not to break *P. falciparum (P. [space] falciparum*) malaria is treatment with ACTs [26]. The use of artemisinin monotherapies in subtherapeutic doses for over 30 years and the availability of substandard artemisinins are thought to be a major driving force in the selection of the resistant phenotype in these regions [9, 10, 33]. Moreover, some therapies declared as combinations in effect have been proven to be artemisinin monotherapies [9]. Resistance to artemisinin compromises the efficacy of the ACT and adds pressure on the partner drug [26].

Resistance to chloroquine has also been confirmed in *Plasmodium vivax* in 10 countries [36]. ACTs are now recommended for the treatment of chloroquine-resistant *P. vivax,* with the exception of treatment with artesunate and SP, where resistance to the partner drug may compromise the efficacy of the combination therapy.

4.3. Economic burden

The use of poor-quality antimalarials can have a negative financial impact on patients and their families [3, 16, 40]. Replacement or additional drugs, repeated courses of poor-quality antimalarials, repeated consultations at health facilities, and lost work days can all impose an unwanted economic burden [3, 16, 40], Indeed, recent modeling assuming current incidence rates of malaria indicates that widespread artemisinin resistance can be expected to lead to 116,000 excess deaths annually [41]. This burden may be particularly severe in some developing countries, where the majority of the population has to pay for their medicines and the cost of the medication represents a substantial proportion of the household income [40].

Poor-quality antimalarials also have negative financial consequences for healthcare systems, pharmaceutical companies, governments and societies, and their use may potentially jeopardize the investments made in the past decades to control and eliminate malaria [3, 16, 40]. The lost productivity and increased healthcare costs associated with the use of poor-quality antimalarials generally occur in resource-constrained countries that are already disproportionately bearing the global cost of malaria [36, 40]. These countries generally lack the increased financial resources needed to inspect, analyze and police the antimalarial supply chain [7].

In particular, the development and spread of resistance to antimalarial medicines has increased the cost of controlling malaria [36, 40, 41]. A decision-tree model estimated the direct medical costs for the effective treatment of malaria using ACT in nonresistant areas to be $US114 million (2013 costs). In comparison, it was estimated that these costs would increase by 28% if ACT was failing at a rate of 30%, and treatment of severe malaria reverted to quinine [41]. Productivity losses were estimated to be $US385 million for each year during which failing ACT was

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used as first-line therapy [41]. Most of this cost was associated with the lost productivity associated with excess morbidity after treatment had failed.

The development of resistance to all current antimalarials would necessitate the development of new, and potentially more expensive, alternatives by pharmaceutical companies, further adding to the economic burden they incur when a poor-quality alternative is used instead of their legitimate high-quality product [40].

If clinical trials use poor-quality medicines, not only are resources wasted, but patients may be harmed [42]. In addition, the erroneous conclusions reached in these trials may inappropriately inform public health policy [42].

5. Tackling the problem of poor-quality antimalarials

A wide variety of issues have contributed to the proliferation of poor-quality medicines in developing countries (**Table 2**) [2, 3]. In order for the quality of antimalarials to be assured, these issues need to be addressed.

Inadequate testing of quality
• Lack of inexpensive surveillance testing systems
• Lack of prequalified laboratories
Poor consumer and healthcare worker knowledge about product authenticity
Inadequate standardization of procedures within quality surveys
Self-prescription of drugs
Availability of products via the Internet
Expensive drugs with large profit margins
Trading in free-trade zones or free ports with minimum regulation
Poor or inadequate national, regional and global legislation, with few legal penalties
Absence of drug regulatory authorities
Unethical practices by manufacturers of antimalarials
Lack of political will and cooperation from stakeholders
Stockouts, thefts, and the erratic supply of antimalarials

Table 2. Factors contributing to the manufacture and distribution of poor-quality antimalarials [2, 3, 16].

It is essential that antimalarial medications be produced according to good manufacturing practice, contain the correct drug(s) and excipients in appropriate doses, and have bioavailability that is similar to the reference product; in addition, the drugs must be stored under appropriate conditions, and be dispensed before their expiry date [26].

Some of the factors that contribute to the availability of poor-quality medicines can only be addressed by collaborative action between law enforcement bodies, regulatory authorities, and customs and excise agencies [26]. Encouragingly, some initiatives and mechanisms are already in place to help ensure that quality assured antimalarials are available for use in malaria-endemic countries.

5.1. Detection methods and technology

Tests capable of accurately detecting and classifying poor-quality medicines at the point of entry into countries and at public and private pharmacies are essential for understanding the types, names, extent, and amount of poor-quality medicines being dispensed both nationally and globally [2, 7].

Such tests can be conducted using quick, inexpensive methods in the field with the aim of examining packaging and detecting drug contents [7]. These tools would also empower those inspecting the medicines throughout the supply chain [7]. Rather than having to wait for the samples to be sent to national or international laboratories, results would be available more or less immediately [7]. Current methods for testing the quality of drugs in the field include visual packaging inspection, lot number reporting via mobile phones, thin-layer chromatography, colorimetric tests, and simplified spectroscopic methods [2, 7]. Portable instruments used to screen for the quality of antimalarial agents include Raman spectrometers and the Global Pharma Health Fund GPHF-Minilab® [40]. Other simple, technologies for testing the quality of antimalarials in the field are in development [43, 44].

More accurate methods of testing the quality of medicines involve laboratories that are equipped for exhaustive chemical analysis [2]. Such investigations can be difficult in developing countries, given the sophisticated and expensive equipment, the type of reagents, and the level of technical expertise that are required. For example, Kenya, Tanzania, South Africa, and Uganda are currently the only countries in Africa with WHO prequalified laboratories for drug quality testing [19, 45].

5.2. Field reports

Quality surveys can serve as an important source of information about the quality of medicines available. Data on the quality of antimalarials, if properly collected, interpreted and used, are essential for the planning of effective interventions [46]. However, reports that have not employed rigorous scientific techniques will potentially bias results [47]. To ensure consistency and accuracy, the WHO has recently produced draft guidelines for the conduct of surveys of quality medicines [46].

5.3. Legislation and regulation

Existing laws in many countries are insufficiently strict to deter the manufacturing and distribution of poor-quality medicines; where this legislation exists, it may not be implemented [22]. Such absence of legislation prohibiting the manufacture and distribution of poor-

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quality medicines encourages their continued manufacturer, since there is no fear of being apprehended and prosecuted [2, 22].

Appropriate national medicine regulatory authorities would help to ensure that all pharmaceutical products on the market were safe, effective and meet approved quality standards [48]. Such regulation would control registration and postmarketing surveillance (quality monitoring and pharmacovigilance) of medicines, as well as the licensing and inspection of manufacturers, importers, exporters, wholesalers, distributors, pharmacies and retail outlets, control of clinical trials, and control of the promotion (**Figure 2**) [48]. It has also been suggested that an international legal convention against poor-quality medicines would address both regulatory and criminal international governance challenges [7].

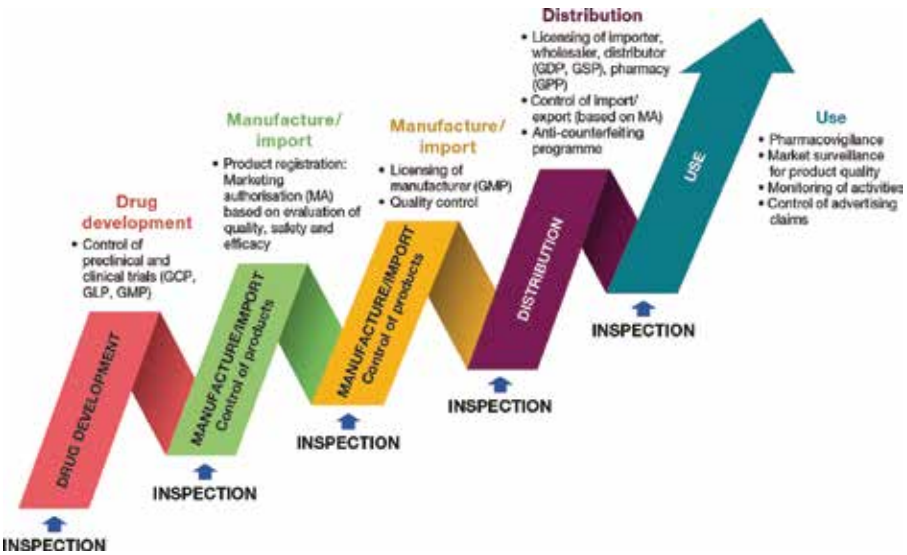

Figure 2. Regulatory system for medicines [48]. GCP, good clinical practices; GDP, good distribution practices; GLP, good laboratory practices; GMP, good manufacturing practices; GPP, good pharmaceutical practices; GSP, good storage practices.

However, the WHO has estimated that 30% of member countries lack any capacity to oversee medicine manufacture, importation, or distribution [23]. At present, only about 20% of member countries have well-developed medicine regulation at varying levels of development and operational capacity.

In particular, the lack of global harmonization of drug registration processes is contributing to the increased availability of poor-quality antimalarials. Regulatory authorities such as the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) provide guidelines for the registration of new drugs for the pharmaceutical industry. Such guidelines include steps for assuring drug quality [49, 50]. For example, the FDA regulates and controls new drugs according to the new drug application process and the

review and ultimate approval of generic drugs is controlled by regulations for abbreviated new drug applications.

However, similar rigorous standards are not applied universally, and particularly in sub-Saharan Africa. Africa currently has more than 50 independent local regulatory health authority agencies, with different administrative and technical requirements, processes and timelines for medicine registration and regulatory review, and with variable transparency of the registration process. A recent WHO assessment in 26 sub-Saharan African countries found that drug approval regulation was not carried out to the extent required to ensure the quality, efficacy, and safety of medicines [48]. Few countries in sub-Saharan Africa relied on the decisions made by other regulators, such as those stringently applied by the EMA or FDA, or by the WHO through its Prequalification of Medicines Program [48].

5.4. The role of the WHO

Many look to the WHO for leadership in regulating the quality of medicines, especially in light of its successful implementation of the public health treaty on tobacco control [7].

Due to the association of oral artemisinin monotherapies with the development of resistance, the WHO urges regulatory authorities in malaria-endemic countries "*to take measures to halt the production and marketing of these oral monotherapies, and promote access to quality-assured artemisinin-based combination therapies*" [51]. The number of countries that allow the marketing of oral artemisinin monotherapies has dropped markedly since the World Health Assembly adopted a resolution supporting the ban in 2007. As of November 2015, marketing of artemisinin monotherapies was still allowed by seven countries: Angola, Cape Verde, Colombia, Gambia, Sao Tome and Principe, Somalia, and Swaziland [51]. However, a continued strengthening of pharmaceutical regulation and the enforcement of existing regulations will be required for the complete withdrawal of oral artemisinin monotherapies from all countries.

In an attempt to standardize the quality of medicines that reach the market, in terms of their efficacy, safety, and method of manufacture, the WHO are managing the Prequalification of Medicines Program [24]. This program's quality evaluation criteria are based on international pharmaceutical standards and a mix of the best practices applied by the world's leading regulatory authorities [52]. Products that meet all of the criteria of the WHO Prequalification of Medicines Program are considered to be quality assured and are added to a WHO list of prequalified medicinal products [53]. This list is used by international procurement agencies and by countries to guide bulk purchasing of medicines. Currently, over 40 of the prequalified medicinal products on this list are antimalarial agents [53]. This program has validated the entry of a number of generic products into markets and procurement pools [52]. Such validation has provided an incentive for other manufacturers of generic products to raise their quality standards and increase the availability of affordable medicines.

In addition to prequalifying medicines, this program also prequalifies pharmaceutical quality control laboratories and active pharmaceutical ingredients, as well as advocating for medicines of guaranteed quality [24].

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5.5. The AMRH initiative

One of the means of improving quality standards is to engage regional networks in developing countries with larger, more powerful and better resourced organizations such as the WHO. One example of such a network is the African Medicine Registration Harmonization Initiative (AMRH) [25, 54]. This program aims to harmonize the registration of medicinal products in Africa across regional economic communities (RECs) [25, 54]. In Africa, the RECs range in size from five to more than twenty-five member states—and all have been invited to participate in the AMRH program.

Partners involved in the AMRH program include the WHO, the New Partnership for Africa's Development, the African Union Commission, the Pan-African Parliament, the World Bank, the Bill and Melinda Gates Foundation, the UK Department for International Development, and the Clinton Health Access Initiative [25]. The WHO is to provide leadership for the development of common technical standards, documents, tools, and processes in line with international standards. The WHO also provides technical assistance for capacity-building and organizes joint assessment and inspection activities [25].

The first REC to secure funding from the AMRH initiative trust fund was the East African Community (EAC). The EAC Medicines Registration Harmonization (MRH) project was formally launched in 2012 in Tanzania and aims to achieve a harmonized medicines registration process in its member countries (Uganda, Kenya, the United Republic of Tanzania, Rwanda, and Burundi) based on common documents, processes, and shared information [25]. Given the progress made in the EAC MRH project, the AMRH initiative plans to expand to other RECs in Africa. It is hoped that five or six groupings will eventually cover the entire African continent. Continued support by concerned governments and international partners will be crucial for its success [25].

5.6. The role of pharmaceutical companies

Poor manufacturing and quality-control practices in the production of genuine drugs (either branded or generic) have considerable impact on the quality of medicines [40]. Pharmaceutical companies are responsible for ensuring that quality meets the guidelines of stringent health authorities, for testing their medicines accordingly and for releasing only medicines that pass these requirements [55]. Chinese and Indian manufacturers are commonly cited as sources of poor-quality antimalarials, but manufacturers in other countries may also be involved [7, 40]. It is essential that pharmaceutical companies behave ethically at all times and follow relevant guidelines and codes of conduct provided by regulatory authorities if the quality of medicines is to be assured.

Reputable pharmaceutical companies must ensure that medicines are under strict surveillance, and with quality control measures in place at all stages of the drug supply chain. Novartis, for example, ensures the quality control of medicines in a three-step process that involves verification, authentication, and pharmaceutical forensic investigations. Verification occurs at the local level to attest to the genuineness of the packaging materials and the plausibility of the manufacturing data (batch number, manufacturing, and expiry date). Authentication, done

in the field, involves analyzing the product (tablets, liquids) with vibrational spectrometers. In the case of substantiation of falsification of medicines, a pharmaceutical forensic investigation then occurs, and any substantiated falsification incidents are escalated to the key stakeholders and national health authorities and the WHO are notified.

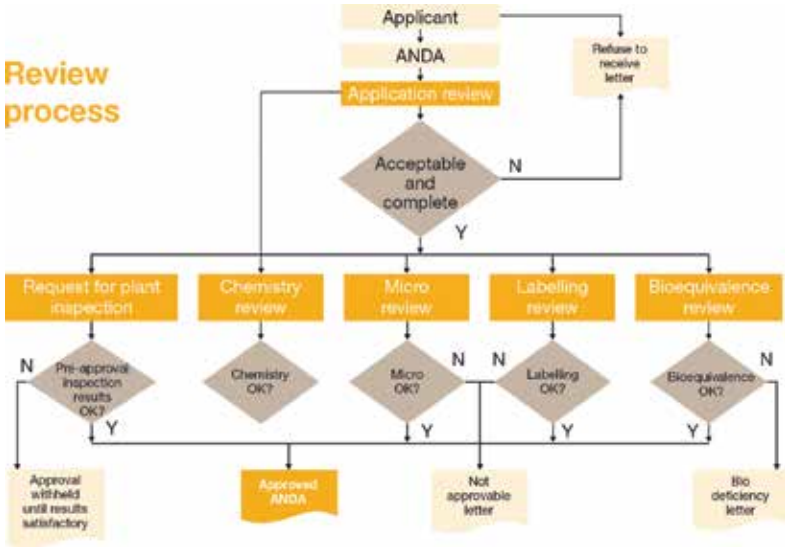

Figure 3. Process for review and approval of generic drugs (ANDA) [58]. Source: US Food and Drug Administration (public domain).

In countries where stringent regulatory authorities exist, pharmaceutical companies developing new drugs must follow a new drug application process that requires both preclinical and clinical studies [50]. In contrast, the approval of generic drugs or new formulations of existing drugs occurs via an abbreviated process that does not require clinical efficacy and safety studies (**Figure 3**) [56]. One of the dangers with some generic products is that they may contain the correct amounts of the drug, but, because of their formulation, they are not adequately absorbed, resulting in lower efficacy. Consequently, this abbreviated process requires proof that the generic product (or new formulation of a reference product) and the reference product have comparable bioavailability. If this is established, the products are said to be bioequivalent [56, 57].

Different types of evidence may be used to establish bioequivalence for pharmaceutically equivalent drug products, including in vivo or in vitro testing, or both [56]. If a generic product or new formulation shows equivalent exposure to the original marketed compound, it is assumed that its efficacy and safety will also be equivalent [56]. To this end, the WHO recommended that not only must all antimalarial medicines be manufactured according to good manufacturing practice and have the correct drug and excipient content, but they must also be proven to have bioavailability that is comparable to that of the reference listed product [26].

http://dx.doi.org/10.5772/64671

An example of a study that was conducted in line with these bioequivalence guidelines involves the antimalarial artemether-lumefantrine [58]. Because development of this compound adhered to these guidelines, this formulation received the WHO prequalification status in June 2014 [53]. The novel fixed-dose tablet formulation of the antimalarial artemether-lumefantrine 80/480 mg was developed in order to reduce the pill burden at each dose and potentially enhance adherence. It was then tested in a bioequivalence study in healthy volunteers against four standard tablets of artemether-lumefantrine 20/120 [59]. Bioequivalence between the two formulations was established, with the prespecified criteria for bioequivalence being met. These prespecified criteria were that the 90% confidence intervals for the geometric mean ratio (novel formulation versus four standard tablets) of the primary pharmacokinetic endpoints (AUC and C_{max}) for artemether and lumefantrine were contained within the acceptance interval of 0.80–1.25. These outcomes established that the rate and extent of absorption of both components of the ACT in the novel formulation of artemether-lumefantrine are comparable to those in the standard tablets.

6. Conclusion

There is a pressing need to reduce the use of poor-quality antimalarial medicines, especially in Africa and Asia. The problem is driven by several factors, including a lack of national and international drug legislation, and inadequate quality assurance by some pharmaceutical companies. This chapter has reviewed the steps and studies needed to ensure development of high-quality medicines based on health authority regulations (including WHO prequalification). The problem of poor-quality medicines can only be addressed by a multipronged approach that includes tackling poor regulation and ineffective/poorly implemented laws at national and international levels. Sustained national and international financing must underpin any such future approach. A legal framework or treaty that protects all countries against poor-quality medicines is also urgently required. Such a framework would facilitate the production of high-quality antimalarials and protect all countries against those who produce, distribute, and sell poor-quality products. In addition, pharmaceutical companies must be responsible for ensuring that the quality of antimalarials meets the stringent guidelines established by regulatory authorities, for testing their medicines accordingly and for releasing only medicines that pass these requirements into the market. The harmonization of the registration process across all nations would help ensure that patients throughout the world obtain access to high-quality antimalarials.

Surveillance of the quality of antimalarials is an essential component of the global fight against malaria. The establishment of a highly qualified, well-resourced international organization that works in collaboration with national medical regulatory bodies, pharmaceutical companies, and international agencies may help to ensure access to high-quality antimalarials on a global level. The implementation of strategies such as the WHO Prequalification Program, the AMRH initiative, and the ethical production of medicines by pharmaceutical companies will help to ensure that antimalarial therapies marketed in low-income, malaria-endemic countries are quality assured. Future research must focus on innovative technologies that accurately and

affordably support the detection of poor-quality medicines at all levels of the supply chain, including prequalified reference laboratories. It is to be hoped that the implementation of such a multipronged approach by policy makers and leaders at the international and national levels will ensure the continued global availability of affordable, high-quality drugs to patients with malaria.

Acknowledgements

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Vaccines

Chapter 15

The Next Vaccine Generation Against Malaria: Structurally Modulated *Plasmodium* Antigens

José Manuel Lozano Moreno

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65251

Abstract

Challenges for obtaining more effective malaria vaccines depend on precise selection of antigenic motifs and understanding the complexity of *Plasmodium* spp. life cycle. Naturally expressed antigens are characterized for being weak immunogenic when tested as vaccine components, thus these have to be strategically modified to render them immunogenic. A molecular clue in this pursuit is provided by the chemical peptide-bond processing by peptidases, which follows a multistep pathway including ephemeral high energy molecular complexes known as transition states. Thus, we have proposed non-natural peptide-bond isosteres as transition states mimetics, and therefore, stabilizing these high-energy states with site-directed designed immuno-mimetics have demonstrated being a rational approach for stimulating antibody populations harboring multiple functional capacities. Therefore, peptide-bond substitutes constitute a coherent pathway towards obtaining selected immuno-active compounds from specific plasmodial molecular objectives. Chemical strategies for synthesizing peptido-mimetics and antimalarial selected trials lead us to assess a number of peptide-bond substitutes for obtaining immuno-active and structurally defined molecules. *Plasmodium* antigens expressed on merozoite, sporozoite and gametocyte stages have been selected as targets and subsequently modified based on the presence of either a high-binding motif or a potential HLA-reading frame. This new family of immuno-mimetics is and efficient neutralizing antibody inducers when tested in *in vitro* and *in vivo* experiments, thus representing a new generation of malaria vaccine components.

Keywords: malaria, synthetic vaccine, non-natural elements, immuno-mimetic, functional antibody

1. Introduction

Malaria is spreading from old *Plasmodium* colonized territories to other zones of the earth where this lethal disease did not exist before covering about 45% of the planet. Over decades an important number of public health trials for malaria eradication and control have been conducted with a limited success. Malaria is caused by *Plasmodium* spp. to a susceptible human being and is transmitted by the *Anopheles* female mosquito bite and is responsible of 660,000 deaths and around 219 million new cases annually (a range of 154–289 million) especially in children younger than 5 years of age and pregnant women inhabitants of endemic and high transmission areas [1–3].

A number of difficulties inherent to many causes among the pathogen resistance to antimalarials, poor coverage of public health programs and natural human host genetic restrictions make the finding of an effective malaria vaccine be an urgent need.

Malaria vaccine development has constituted a big challenge for researchers, up-to-date about 236 vaccine candidate prototypes are being tested. Approaches based on strategies such as immunization with irradiated sporozoites of *Plasmodium* spp., as well as DNA-based immunogens besides recombinantly expressed antigens such as RTS,S formulated in different vehicles and strong adjuvant systems and prototype delivery systems such as virosomes besides to the most promising strategy constituted by synthetic peptides representing subunit multistage immunogens formulated in human allowed adjuvants represent the main attempts for immuno-prophylaxis [2].

Plasmodium spp. express a number of antigens, more than 100 in its different life cycle stages and most of them have been regarded as vaccine targets such as the so-named merozoite surface antigens 1–10 (MSP1–MSP10), erythrocyte binding antigens EBA-140 and EBA-175, the ring-infected erythrocyte surface antigen (RESA-155), the apical membrane antigen AMA-1 among many others from the merozoite stage including a number of organelle's proteins. Besides the circumsporozoite surface protein (CSP), sporozoite threonine and asparagine-rich protein (STARP), the sporozoite and liver stage antigen (SALSA), the liver stage antigen (LSA) are some representative antigens of the sporozoite stage and the classical Pfs48/45, GSA and Pbs25/28 from gametocytes have been regarded as the targets for transmission blocking vaccines [4].

Natural immunity to malaria is related to hemoglobin structure, some disorders such as thalassemia confer resistance to *Plasmodium falciparum* while Duffy negative RBC constitutes the mechanisms associated to malaria resistance to *Plasmodium vivax*. Immune response against a natural malaria infection is nonspecific and has a weak effect on protection. Innate immunity mediated by NK and non-related auto antibody B-cells as well as the INF-gamma response to infected red blood cells act as a primary line of defense against *Plasmodium* infection. In the adaptative immunity step $CD4^+$, dendritic cells, macrophages, gamma delta T cells and NKT cells are able to detect the parasite and participate in the immune defense. Besides natural immunity to this disease, an effective malaria vaccine can be proposed for a strong stimulation of antibody B-cells. However, at is has been demonstrated that most *Plasmodium* native-derived

http://dx.doi.org/10.5772/65251

sequences are proven to be poorly immunogenic and non-protection inducers against malaria. In order to understand this problem, Patarroyo have established a strategy based on selection of non-polymorphic regions of selected antigens and a subsequent rational mutation of those residues belonging to red blood cells and hepatic cells binding motifs allow modified active antigens [4], however, most clinical trials worldwide which have been performed with the above mentioned malaria vaccine candidates have failed to achieve significant protection levels, evidencing intrinsic difficulties for developing a potent fully protective vaccine formulation. Perhaps pathogens including *Plasmodium* spp. have evolved complex mechanisms to recognize, block and destroy natural-presented antigens as vaccines as well as others related to antigen structure modulation.

Our group has introduced in this pursuit some non-natural elements to be incorporated into synthetic antigens with the aim of governing both antigen presentation as well as specific B-cells for functional neutralizing antibody stimulation. Some of these non-natural elements included capture sequences for stimulating antigen degradation and others for the peptide-bond structure modulation. Peptide-bond isosteres included reversal configuration thereof, urea motifs and reduced amide as peptide-bond surrogates all constituting a novel immunogen family herein named as immuno-mimetics.

Once strategically incorporated into selected antigens, produced peptide-bond surrogates overcome non-desirable properties of native non-modified antigens such as cytotoxicity and hemolytic profiles, besides prolonging these new molecules half-life and a remarkably strong immuno-stimulating activity that can be associated to the newly introduced freedom degrees to the 3D structure of immuno-mimetics.

Also, we have consolidated the female BALB/c animal model for malaria vaccine candidate testing based on controlled challenging performed to immunized animals with two rodent malaria strains, being those *Plasmodium berghei ANKA* and *Plasmodium yoelii*-17XL. Additionally, passive transferring antibodies into infected animals have proven to be efficient for malaria disease control and parasite clearance.

2. Global health statistics, economical and environment determinants

In 2015, time for fulfillment of the millennium development goals (MDGs) was getting closer to the end, and a consequent protocol comprising 17 sustainable development goals (SDGs) constitute the next step. In its annual report, World Health Organization (WHO) analyzed 15 years of advances of those proposed MDG and evaluated the next challenges for the coming years.

As reported in world health statistics 2015 issued by the WHO [1], undernutrition was the main cause of mortality in an assessed 45% of all deaths of children under 5 years of age. In the 1990–2013 period, the estimate of underweight children in third-world countries decreased from 28 to 17%, and a sustainable decreasing rate to 16% was expected for the end of 2015. In spite of those proposed efforts for achievement of MDG, these numbers are not still sufficient

to the goals. The proportion of underweight children declined globally from 25% in 1990 to 15% in 2013.

Worryingly, poverty is strongly associated with public health especially to problems related to high transmission of infectious diseases. As observed in **Figure 1A**, among the main causes of deaths among children under 5 years (in neonatal and post-neonatal ages), between 2000 and 2013 are responsibility of infectious diseases, pneumonia, malaria, HIV/AIDS, measles, diarrhea and sepsis are the main reasons of mortality accounting 13 and 35%, respectively. Malaria represents 7% of children mortality mainly in the post-neonatal period between 1 and 59 months of age.

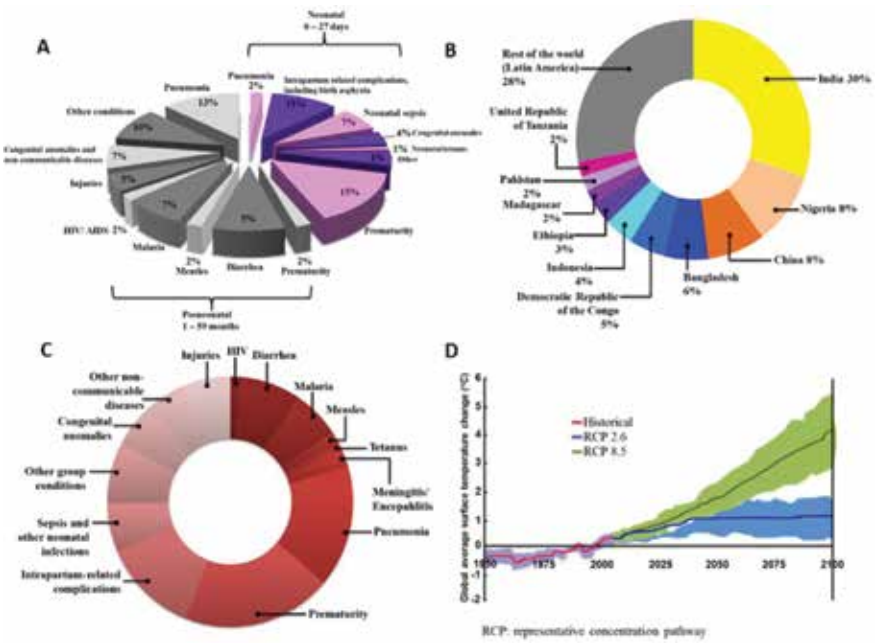

Figure 1. Health statistics and world climate. (**A**) Causes of deaths among children under 5 years of age. **(B)** Top 10 countries with largest share of the global extreme poverty. **(C)** Main causes of child mortality due to transmissible diseases. 5.9 million children under age five died in 2015, nearly 16,000 every day. **(D)** Climate changes and greenhouse effect. Global average surface temperature changes under two scenarios for considering the global greenhouse gas emissions between years 1950 and 2100. RCP for representative concentration pathway. This figure has been adapted from information provided by WHO [1–3].

In the last year, 836 million of the world population lived on less than US$1.25 daily in comparison with 1.9 billion in 1990. In those the so-named poor countries, 14% of the people lived on less than US$1.25 daily in the same year, regarding the 47% in 1990. Getting closer to an amount of US$2 daily has been difficult at higher poverty levels.

The most inhabited counties of the world such as People's Republic of China and India have been crucial for world reduction in poverty (indeed India remains the earth's country having most extreme poverty; **Figure 1B**) such reduction can be associated to growth of central

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economic sectors and labors. Other factors such as income transferring, remittances and evolving new demographic profiles have had a lesser impact. However, those efforts have not been enough since one of each seven people in poor countries live on less than US$1.25 daily. In the sub-Saharan countries, more than 40% people are living in extreme poverty in 2015. In the countries having middle-incomes, the 73% of the Earth's poverty is found [3].

Figure 1B displays the top 10 countries with largest share of the global extreme poor, accordingly with WHO classifications, these countries are inhabited by people living on less than US $1.25 per day. Therefore, poverty levels show India 30%, Latin America 28%, China 8% and dramatically 20% represents African countries (Nigeria, Democratic Republic of the Congo, Ethiopia, United Republic of Tanzania, among others). Child mortality was 5.9 million children under age five which died in 2015, nearly 16,000 per day, mainly caused by infectious diseases whose distribution can be observed in **Figure 1C**. Main causes of child death are due to measles, malaria, diarrhea, HIV/AIDS, meningitis/encephalitis, tetanus and sepsis and other neonatal infections besides prematurity among other causes [1–3].

The sustainable development goals (SDGs) also contain ambitious targets for child mortality, with SDG 3.2 seeking to end preventable deaths of newborns and children under five. Those have included local aims for reducing the under-five mortality rates (U5MR) around to 25 deaths per 1000 live births as well as the neonatal mortality rate (NMR) to lower than 12 per 1000 live births, in comparison with a world's U5MR rate of 43 per 1000 live births in the last year, representing 5.9 million deaths of children under 5 years and a NMR rate of 19 per 1000 live births, representing 2.7 million deaths in the first month of life. Main causes of newborn mortality during the last year were prematurity, birth-related complications and neonatal sepsis, while those post-neonatal causes of death were associated to pneumonia, diarrhea, injuries and malaria. Specifically, the so-called Target 4.2 in the document, which encourage for assuring that most children have access to good quality development, heath assistance and care, and basic education join to reducing child mortality while improving a better living quality for childhood in most poor countries [3].

On the other hand, **Figure 1D** displays climate changes and greenhouse effect on earth for a period between 1950 projected to the wear 2100. As described, global average surface temperature change is estimated under two scenarios for turning around global greenhouse gas emissions [1].

The global climate warming is a reality. The average data for the Earth's surface temperature showed a 0.85°C increasing (0.65–1.06) for the 1880–2012 period. Data show that the Earth's north hemisphere had the warmest period from 1983 to 2013, being the highest regarding the last 1400 years. Without any doubt, most causes for this fact can be associated to human activities. Mathematical and predictive algorithms for global warming-cooling allow establishing precise predictions on climate changes over long-time periods, these have included factors such as volcanic activity and gas emissions to the atmosphere. The Intergovernmental Panel on Climate Change's (IPCC) for temperature changing prediction have considered a number of factors and possibilities for future greenhouse gas emissions, which have been termed as representative concentration pathways (RCP). This ranges from RPC 2.6 which considers that global greenhouse gas emissions will reach a highest value between 2010 and

2020, then it significantly decrease after 2020, to RCP 8.5, in which greenhouse gas emissions will continue to increase during the present century. Middle-range positions consider that RCP 4.5 and 6.0 would reach the highest emission values in 2040 and 2080 in consequence [1].

World's predictive temperature changes for 2015–2016 period regarding those recorded between 1986 and 2005 are estimated to vary between 0.3 and 0.7°C. Similarly, increasing temperature ranges for the 2081–2100 period regarding the recorded changes between 1986 and 2005 has been estimated to be 0.3–1.7°C (RCP 2.6) to 2.6–4.8°C (RCP 8.5) (**Figure 1D**). In consequence, the Arctic region's warming rate will increase faster than the world's mean, and that for the land's rate will be higher than the mean for the ocean. Assessed RPCs led to estimate that sea level will growth from 0.26 to 0.82 m by the final of the current age. Earth's surface warming and climate variations will have a deep impact on human living, health and welfare, since obtaining drinking water and the possibility of cultivating the necessary quantities of agricultural products and all resources required for the future world's larger population will be compromised.

3. Malaria: a devastating disease

Malaria is a global disease responsibly of high levels of morbidity and mortality especially in developing countries whose inhabitant populations suffer the consequences of the disease besides the economic impact on these populations. At the beginning of the new millennium, a global strategy for controlling malaria by establishing a global founding for fighting three high impact diseases, i.e., AIDS, tuberculosis and malaria have been proposed by the World Health Organization (WHO) [5]. The 2015 world malaria report from WHO account data from 79 countries affected by this disease reflecting a slight improvement in controlling the disease impact but the problem still remains for a solution. In 2013, diagnosis tests were expanded to most malaria affecting countries and huge steps towards vector control were also conducted. In 2013, the use of insecticides impregnated mosquito nets were promoted and so the amount of populations protected against malaria were increased, thus mortality due to malaria was reduced to 47% between the years 2000 and 2013. However, endemic areas are still far away from reaching a total coverage for malaria control and available founding is each time decreased for managing this important problem. An estimated 278 million people in Africa live in households without a single insecticide mosquito net and 15 million pregnant women have no access to a preventive treatment for malaria. In addition, other diseases affecting these populations alter the development of related campaigns is the case of Ebola whose recent outbreak have conducted to a decreasing in health assistance in those affected zones.

In the last five years, it is estimated that 584,000 deaths due to malaria have occurred (367,000–755,000) of which 78% were children under 5 years of age and 90% came from Africa; today, there are an estimated 3.2 billion people at risk of contracting the disease since are living in areas influenced by the disease, of which 1.2 billion are at high risk (more than 1 into 1000 possibility of acquiring malaria in the year); in the Region of the Americas, it is presumed that the risk is 120 million people in 21 countries in the region [5].

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Eradication efforts by public health preventive measures are not sufficiently effective for many reasons, among which are the socioeconomic, demographic and technical policies, emerging resistance to insecticides by the vector and to antimalarial drugs by the parasite [6]. In 2010, vector resistance had been reported in 49 countries around the world of which 39 reported resistance above two or more pyrethroid insecticides. In 2013, this report increased to 82 countries reporting insecticide resistance [3], therefore, to develop an effective vaccine against the disease becomes an urgent need.

By 1967, major efforts were made to find an effective vaccine against human malaria, in one of the most important related studies of the time, 59% protection was achieved after an intravenous challenge of a malaria murine model after being vaccinated with 75,000 live attenuated irradiated sporozoites [7].

Currently among vaccine candidates that are in more advanced clinical trials are the RTS,S and *Pf*SPZ which incorporates the use of non-replicative attenuated sporozoites through controlled radiation [8], which has obtained a dose dependent protection in humans being necessary the application of 1.35×10^5 attenuated sporozoites in five doses [9]. It should be noted that the duration of protective antibodies has not been fully established, thus obtaining a vaccine is not a reality.

As can be observed in **Figure 2**, global malaria spreading accounts for more than 80 countries that are affected by malaria infection (purple background in the map). Besides, insecticide susceptibility status for malaria vectors (*Anopheles* female mosquitoes) demonstrates a resistance increasing to most insecticides concomitant with areas of high transmission of malaria.

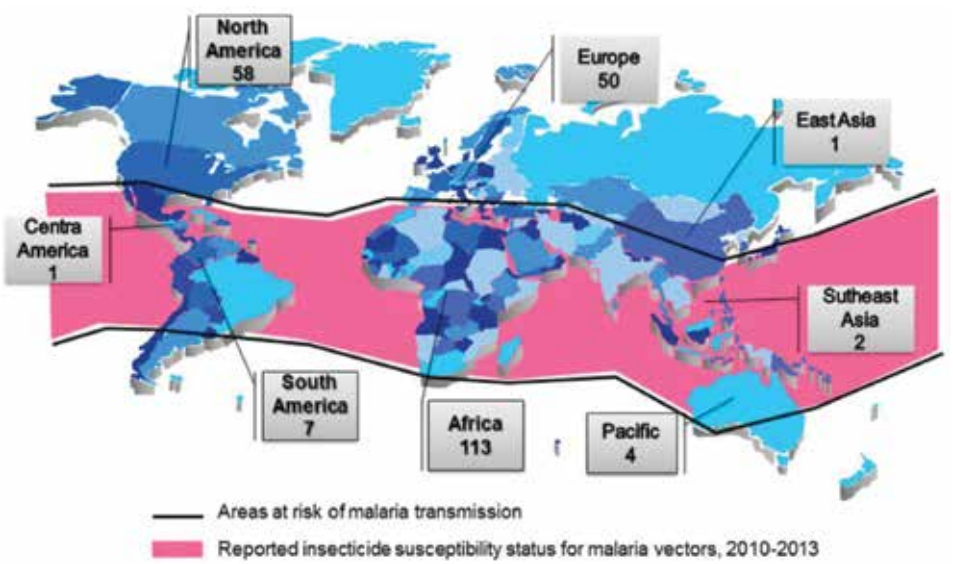

Figure 2. Areas of risk of malaria transmission and ongoing malaria vaccine candidate trials.

4. *Plasmodium* spp. life cycle

A deep knowledge and understanding of the *Plasmodium* parasite life cycle would be a key step towards antigen discovery, and it will establish the molecular basis for a proper immunogen designing to be further tested as a vaccine candidate. The *Plasmodium* spp. belong to the phylum Apicomplexa being the causative agent of malaria, whose clinical and pathological manifestations are associated with asexual erythrocytic stage of the parasite [10].

There are five species of *Plasmodium* causing human malaria, the most lethal disease is caused by *P. falciparum* and followed by *P. vivax*, and less prevalent are *Plasmodium malariae* and *Plasmodium ovale* [11] in 2011 *Plasmodium knowlesi* was included in this list. In Colombia for the year 2014, 356 clinical cases of uncomplicated malaria were reported, 20,074 cases of malaria by *P. vivax*, 19,789 by *P. falciparum*, 17 cases by *P. malariae* and 561 cases of mixed malaria according to data presented by the National Institute of Health (INS) in its weekly report SIVIGILA [12].

Plasmodium parasites have a complex life cycle involving interactions of invertebrates (vector) and vertebrates (mammalian host), besides presenting various stages in intracellular and extracellular environments (**Figure 3**) [13].

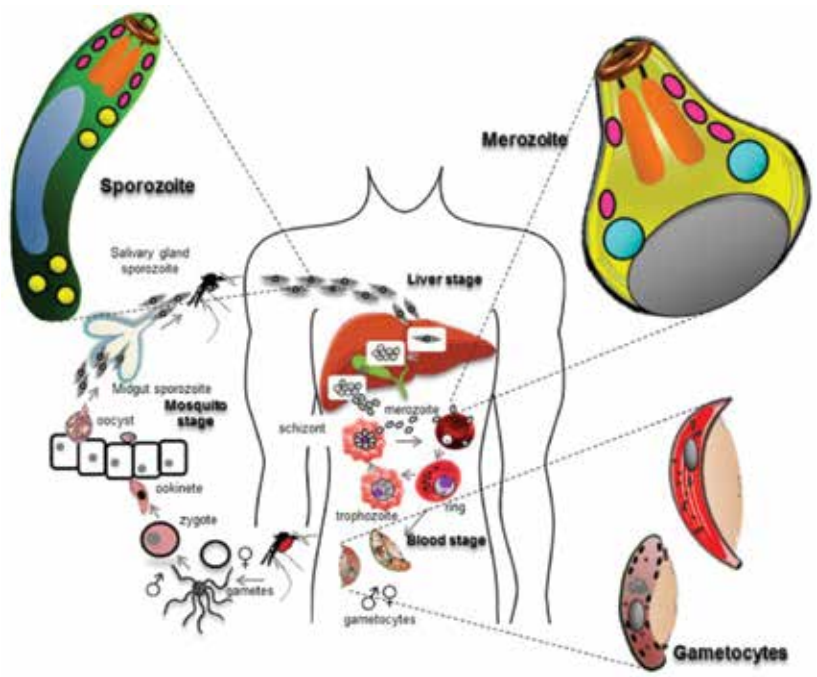

Figure 3. *Plasmodium* life cycle. Main pre-erythrocyte, blood and sexual stages are denoted.

In the human host, sporozoites are inoculated by the bite of female *Anopheles* spp. mosquitoes, then invade hepatocytes in a time between 5 and 30 min; within hepatocytes each sporozoite

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develops a schizont which release between 10,000 ± 30,000 merozoites to blood stream during a period of 2 ± 10 days depending on the parasite specie [14], in the case of *P. vivax* and *P. ovale* also it produces a different stage in the liver called hypnozoite, which is a silent form responsible for the subsequent relapses [11]. During the travel from the skin to the liver, the parasites cross the capillary epithelium in the dermis and enter to blood circulation, cross the hepatic sinusoids epithelium to enter the parenchyma, and this process as the hepatocyte infection are given by activity of the myosin-actin engine located in the plasma membrane of the parasite and its rhoptries, dense granules and micronemes [15].

The invasion of erythrocytes occurs after several steps with multiple interactions between receptor membrane proteins of host cells and parasite protein ligands expressed in its surface as well as in rhoptries and micronemes [16]. The parasite grows and divides in about 72–48 h according to the specie to the schizont stage which contains more than 30 merozoite particles, which are released with the subsequent invasion and replication in healthy erythrocytes [17]. Acquired immune response induced by malaria parasites is complex and varies depending on the level of endemicity, epidemiology, genetic, age of the host, parasitic stage and parasite species. Repeated infections and continued exposure are required to achieve clinical immunity with symptom reduction and reduced number of parasites in an infected individual or inhibition of parasite replication [18].

5. The murine model in the search for vaccine candidates against malaria

The mouse model has been widely used in the study on malaria, and it has been regarded as a practical model for experimental studies since its genetical features regarding human beings such as homology and similarity at the protein structure level, physiology and life cycle besides of owning a malaria transmission vector (*Anopheles stephensi*) that can be maintained under defined laboratory [19].

Due to this, there are several *Plasmodium* strains that infect rodent models by malaria (*P. berghei, P. yoelii, P. chabaudi, P. vincker*), and their experimental behavior can be extrapolated due to the fulfillment of a standard life cycle under controlled conditions. The two most commonly employed strains in malaria vaccine discovery are the *P. yoelii* and *P. berghei*, which have high similarity with the clinical symptoms and pathology developed by *P. falciparum* in relation to those stages of cerebral malaria, placental malaria, severe malaria and organ damage as liver, kidney and lung [20]. The *P. berghei* infection model has allowed demonstrating the role of interferon in response to parasite replication in the liver, and this participation was then demonstrated in *P. falciparum* [20].

The rodent malaria infection by *P. yoelii* has allowed to demonstrate that humans immunized with the *Pf*CS protein from the parasite sporozoite stage, induced antibodies that cross-react with *P. yoelii*, as well as mice immunized with *Py*CSP stimulated antibodies that cross-react with *P. falciparum*; therefore, this model evidenced its usefulness as a predictive tool for immune response against certain malarial antigens since there are a 70% of genome similarity between *P. yoelii and P. falciparum* [14]. This similarity associate at least 3300 orthologous

genes of *P. yoelii* with 5268 genes of *P. falciparum* [21]. Although the erythrocytic cycle of *P. berghei* and *P. yoelii* takes place more rapidly (24 and 18 h, respectively) compared to those developed in *P. falciparum* (36–48 h) and *P. vivax* (48 h), differences in tropism for invasion of reticulocytes in the case of *P. yoelii* and *P. berghei* are not presented in *P. falciparum* and the genetic similarity between these *Plasmodium* is quite important, and so the rate of increase in parasitemia levels is similar during the first 3–4 days after inoculation *in vitro* as well as the parasite growth [22]. Also, *in vivo* conditions and clinical symptoms associated to the disease are presented in terms of fever, malaise, splenomegaly and breathlessness related with red blood cells rupture which differ of symptoms present in infections with other strains such as *P. chabaudi* where in contrast the infection is associated with hypothermia [23]. The production and regulation in the cytokines expression are no exception to the similarity between the mouse model and a malaria human infection since *P. yoelii* and *P. berghei* replicate many events that can be correlated between both of the infection types [24, 25].

In studies at the level of liver infection cycle were found that about 654 (92%) of proteins in *P. yoelii* correlated with orthologous sequences present in *P. falciparum* and 66% of the genes in the *P. yoelii* transcriptome have orthologues in *P. falciparum* [26]. Also in this stage of infection, it has been possible to obtain *in vivo* images in models of infection with *P. berghei* in mice, these have shown details of hepatocyte invasion by *Plasmodium* which were not yet known in humans, as well as the fact that sporozoites can recognize heparan-sulfate proteoglycans besides that *P. yoelii* infection models have been conducted to tests the oxidative stress in the liver induced by infected erythrocytic forms [27].

Bearing in mind, the possibilities offered by murine infection models, we have conducted an important amount of experiments in order to test a variety of chemically modified antigens as potential vaccine components.

In spite of impressive economic and political efforts conducted by WHO and other non-government organizations for malaria eradication and control, based on insecticide treatment of bed-nets (mainly DTT), use of new formulations of artemisinin and other antimalarials for treatment of infected patients and teaching about an appropriate water and environment care to inhabitants of malaria high-transmission areas, among other strategies, malaria still remains as one of the most important health problems for developing countries. Contrary to those expectations, most of these strategies have failed for malaria control, mainly due to novel and powerful biological evolution of antimalarials-resistance mechanisms developed by *Plasmodium* parasites, joint to the continuous mosquito adaptation and colonization of new environments and territories, climate changing and global warming due to non-controlled gas emissions to the atmosphere. Therefore, hopes for controlling this lethal disease are based on developing more efficient preventive strategies and highly potent malaria vaccines.

Up-to-date, about 236 including chemoprophylaxis and malaria vaccines clinical trials are being conducted worldwide, most of them have been completed showing a limited success (as shown in **Figure 2**). Most conducted studies have been focused on vaccine candidates aimed to block three different potential targets, being the transmission-blocking approach the first (gametocyte-derived proteins such as Pf25 and Pf125); secondly, those candidates directed against *Plasmodium* liver-malaria stages (considering proteins such as Circumsporozoite

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surface protein (CSP), liver stage antigen (LSA), sporozoite and liver stage antigen (SALSA), Thrombospondin-related anonymous protein (TRAP) and others) and vaccine candidates directed against malaria blood stages (classical merozoite protein targets are Merozoite surface proteins 1-10 (MSP-1-10), apical membrane antigen-1 (AMA-1), ring-infected erythrocyte surface antigen (RESA-155), serine repeat antigen (SERA), Erythrocyte binding antigen 175 (EBA-175) among others).

As recently mentioned by Birkett in 2015, the European Medicines Agency announced a positive opinion for the malaria vaccine candidate most advanced in development, RTS,S/AS01, which provides modest protection against clinical malaria in all conducted trials, but in spite of its poor efficacy later in 2016, this product was recommended by WHO for large-scale trials in moderate to high malaria transmission areas [28]. As observed in **Figure 2**, 113 trials of pharmaceutical products among antimalarials and vaccine formulations are being conducted in Africa in high-transmission malaria regions by immunizing mainly with modified or attenuated sporozoite NF54 strain malarial parasites or other products such as the so-named biological *Pf*SPZ vaccine all of them formulated on strong adjuvant systems such as AS01 as can be observed in the web site ClinicalTrials.gov, a service of the U.S. National Institutes of Health [29].

Due to the moderate success conducted in the last three decades of researching for finding highly potent vaccines for preventing malaria, the field is open for new ideas regarding the discovery of strategies for developing structurally modulated molecular probes which address the *Plasmodium* complex molecular mechanisms involved in parasite detection, facing the challenge of demonstrating protective efficacy profiles and parasite clearance capacity, so those would enter the pathway of being regarded as components of novel vaccine formulations.

6. Current status of *P. vivax* vaccine progress

Morbidity to malaria outside of the sub-Saharan Africa still remains meaningful causing more than 50% of malaria cases, especially in the Americas and Pacific-Asia where poverty and public health systems are associate to multiple problems. The complex *P. vivax* biology and its ability to differentiate into latent forms called hypnozoites which appear longtime later to produce erythrocyte infective forms, prompt occurrence of macro and micro gametes previous to clinical manifestations are seeming, and thus a short evolution cycle into the mosquito makes useless using standard tools to control *P. vivax*. Simultaneously to decreasing in global incidence, some dramatic changes in pathogen infective species have been reported by *P. vivax* being currently the prevalent *Plasmodium* spp. in those mentioned world regions.

For multiple reasons, the epidemiologic spreading of malaria due to *P. vivax* is being regarded as careless. However, turning on attention to malaria caused by *P. vivax* has to be a priority when thinking in a vaccine against malaria. Most approaches for a *P. vivax* malaria vaccine candidate have considered orthologous sequences among the most predominant *Plasmodium* species as being *P. falciparum* and *P. vivax* especially antigens of both pre-erythrocyte and

erythrocyte stages. Among a number of vaccine candidates, the VMP001/AS01 targets the CSP antigen of *P. vivax*. This has been assessed in controlled human malaria infection (CHMI) studies but proven to be unsuccessful with poor protection capacity. Another candidate, which is a recombinantly expressed on appropriate virus, targets the TRAP antigen and currently is currently in study and another prototype vaccine candidate is based on using the strategy of attenuated sporozoites.

On the other hand, the most focused *P. vivax* erythrocyte-stage antigen is the Duffy binding protein (DBP), which is considered crucial for red blood cell (RBC) invasion; however, the DBP non-conserved character establishes an important hindrance. Importantly, time of protection against malaria would be more relevant for a *P. vivax* vaccine regarding a *P. falciparum* vaccine due to *P. vivax* disease incidence is not focused in given populations as it is for *P. falciparum*. Hepatic *P. vivax*-stages also contribute to reinforce this problem complexity [30, 31].

Therefore, developing potent *P. vivax* vaccines would depend on several key aspects among establishing a continuous *P. vivax*—culture in enriched reticulocyte media or specific growth factors aimed to reproducing parasite infections, also appropriate animal models for vaccine candidate testing and most importantly the right selection of multi antigen formulations in human adjuvants and delivery systems, thus peptido-mimetics would play a role in this pursuit.

7. The meaning of being non-visible to α/β-TCR of T lymphocytes

It is well known the fact that the T-cell receptor sees antigen on the surface of cells associated with an MHC class I or II molecule. Therefore, activating humoral and cell-mediated immune responses requires factors such as cytokines and costimulatory molecules expressed by Th cells. A fine and specific regulation of Th has to be highly regulated in order to avoid any self-reactivity would conduct to auto-immune disorders. In order to ensure the Th-cells activation and regulation, these have to recognize a given antigen that is being presented in the MHC class-II context which is located on an antigen presenting cell (APC) surface. As it is known, these professional presenting cells among macrophages, dendritic cells and B lymphocytes harbor two relevant features: (1) surface expression of class-II (MHC-II) molecules, and (2) recruitment of costimulatory molecules as signals for activation of Th-cells.

Antigen-presenting cells first internalize antigen, and then display a part of that antigen on their membrane bound to a MHC-II molecule. The TH cell recognizes and interacts with the antigen–MHC-II molecule complex on the membrane of the antigen-presenting cell. Immune system is prepared for antigen presentation by stabilizing MHC-II molecules in the endoplasmic reticulum bound to an endogen invariant Ii chain which is later cleaved to a small peptide called class II-associated invariant chain peptide (CLIP) which remains bound to the MHC-II molecule to be then replaced by a given antigen-peptide assisted by a chaperone molecule named HLA-DM in endosomal compartments. Therefore, the antigen-MHC-II bimolecular complex will travel to the APC membrane surface to be presented to T-cell receptors (TCR) of

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T-lymphocytes to establish and stabilize in consequence specific ternary complexes able to trigger $CD4^{+}$TH cell proliferation and so an immune response.

As mentioned one of the main functions of CLIP is to prevent the binding of self-peptide fragments prior to the MHC II localization within the endosome-lysosome, a consensus primary structure of CLIP is ^{87}PVSKMRMATPLLMQA^{101}, which is able to a proper interaction with a HLA-II molecule by anchoring-specific residues to the so-named pockets 1, 3, 4 and 9 of the MHC-II molecule in such a way that its entire structure will remain buried into the HLA-II molecule. The CLIP-HLA-II (CLIP: HLA-DR3) molecular complex is shown in **Figure 4**. As observed, the endogenous peptide is hidden into the presenting HLA-II molecule, and the possibility of being recognized by any TCR is completely abolished, and so an auto-reactive immune response will not take place, thus if a given pathogen can develop immune response evasion mechanisms based on its ligands structure features, it would be desirable to its convenience to resemble the most relevant structure characteristics of CLIP to avoid be recognized by TCRs [32].

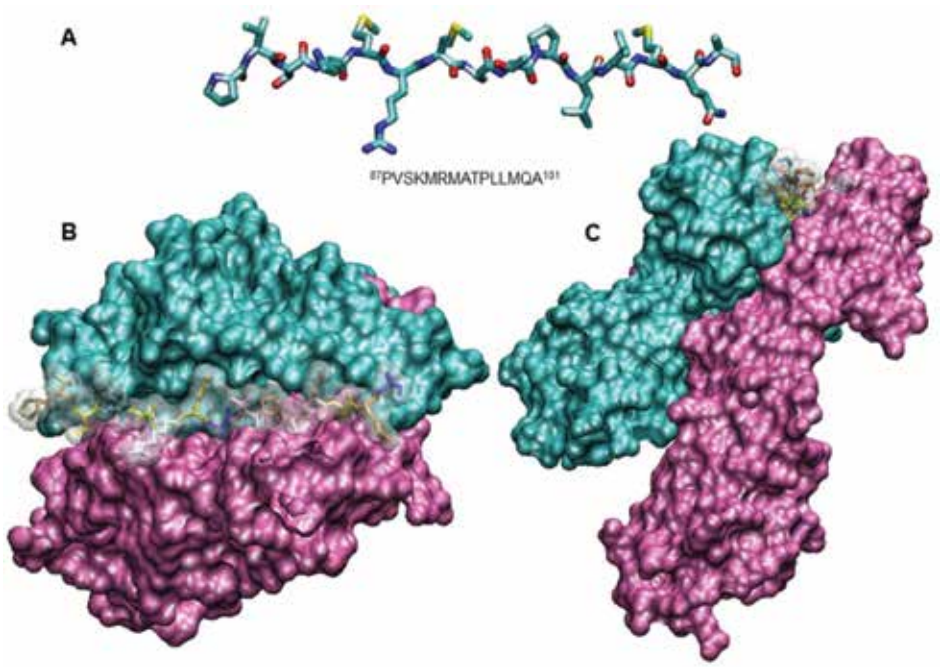

Figure 4. An endogen peptide-MHC-II bimolecular complex. The endogenous invariable Ii-chain cleaved CLIP product (^{87}PVSKMRMATPLLMQA^{101}) complexed to a HLA-DR3 allele. Coordinates of the CLIP-HLA-DR3 complex from the protein data bank (PDB) coded 1A6A corresponded to the structure determined by X-ray diffraction at a 2.75 Å resolution, was downloaded and molecular modeled with the visual molecular dynamics (VMD1.7ή) software of the University of Illinois at Urbana-Champaign. Color code representation for HLAII α-chain in purple, β-chain in cyan, HA peptide is represented in amino acid id code [32].

8. How to become recognized by a TCR-T lymphocyte

Influenza hemagglutinin (HA) or hemagglutinin is a glycoprotein found on the surface of influenza viruses. Its role is to bind the influenza viruses to their target cells through sialic acid, specifically to red blood cells and upper respiratory tract cells [33, 34]. Once the pH has been decreased, a second role of HA is to join the viral cover to formed endosomes. HA is an integral membrane glycoprotein expressed as homo-trimers which seem a barrel-like structure having around 13.5 nm in length. HA is confirmed by three monomers built into a alpha-helical core displaying spherical tips containing those sialic acid-binding motifs. HA is synthesized as monomeric units as precursor forms which are glycosylated and processed on protein maturation, to produce two shorter proteins called HA1 and HA2. The HA monomers are long helical chains attached to the cell membrane by HA2 and capped by HA1. Thus, HA has been responsible for stimulation of neutralizing antibodies which are proven to avoid influenza virus infection to its target cells, thus constituting an important molecular tool for infection control using mechanisms associated to ternary complex stabilization of HA-HLA-II α/β-TCR ($CD4^+$) with specific HLA-DR4 alleles such as DRA*0101 and DRB1*0401 [34].

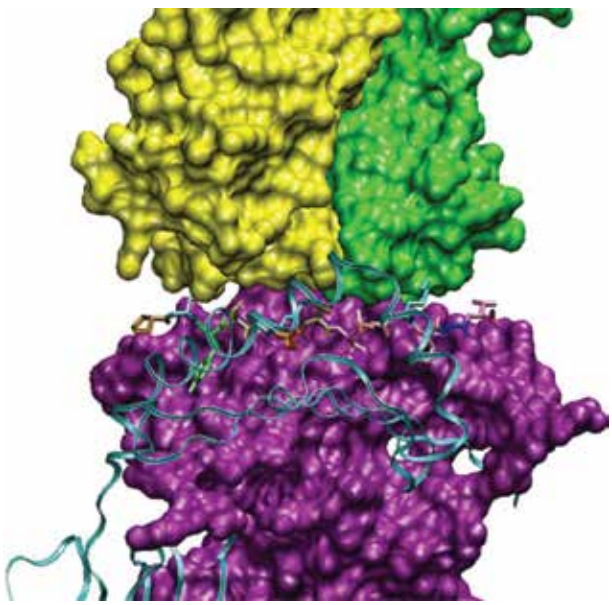

Figure 5. Reactive and T-lymphocyte proliferation upon a stabilized ternary HA-hemagglutinin-HLAII-TCR complex. Coordinates of the human T-cell receptor (TCR) HA1.7, influenza hemagglutinin peptide, and major histocompatibility complex class II molecule, HLA-DR4 (DRA*0101 and DRB1*0401) were downloaded from the protein data bank (PDB) whose code 1J8H corresponded to structure obtained by X-ray diffraction at a 2.40 Å resolution, and then was molecular modeled with the visual molecular dynamics (VMD1.7ή) software of the University of Illinois at Urbana-Champaign. Color code representation for HLAII α-chain in purple, β-chain in cyan, HA peptide is represented in amino acid id code, TCR α-chain in yellow and β-chain in green [34].

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Figure 5 recreates the 3D structure of the HA-hemagglutinin-HLAII-TCR ternary complex. As observed, the HA306–318 peptide backbone whose amino acid sequence is PKYVKQNTL-KLAT anchor-specific residues into the HLA-II 1, 3, 4 and 9 pockets and clearly expose some residues in positions 5, 8 and 7 to be recognized by α/β-TCR chains and so stabilizing the molecular complex. However, the HA peptide binds promiscuously and can be presented by most of the frequently occurring DR alleles. Therefore, $CD4^+$ T-lymphocyte proliferation would lead a subsequent neutralizing antibody production able to block the influenza virus infection, being this molecular interaction an effective mechanism effectively used for the immune response. As this a number of similar immuno-reactive complexes have been described [35–40].

9. New modified vaccine components containing non-natural elements considering chiral and topochemical constraints

Current prototype vaccines against malaria are failed to the goal of protecting any individual living in high risk malaria transmission areas even the most promising such as RTS,S which have less than 30% effectivity. As discussed above, the complex life cycle of *Plasmodium* parasites besides human genetic restrictions and immune mechanisms associated with protection makes obtaining an effective malaria vaccine a challenge. Therefore, obtaining new immunogens as vaccines and other molecular systems having potential application in malaria therapy would have to be with precisely selecting relevant antigens which would be submitted for steps of redesigning, production, formulation and *in vitro* tested before assayed in animal models; thus, after proven to be effective have to cross the border line for human clinical trials.

The aim of our research is to produce back-bone modified immunogenic antigens which included non-natural elements such as chiral and peptide-bond substitutions directed to modulate the antigen 3D structure and stimulation of neutralizing antibodies. Our approach is based on low-polymorphic sequences of *Plasmodium* which are then modified into immunological relevant motifs as well as strategically including lysosomal substrates to stimulate processing and presentation steps on vaccinated individuals. Therefore, we have analyzed and produced representative modified immunogens based on a number of native sequences belonging to different stages of *Plasmodium* which upon vaccination of animal models have proven to be effective against malaria infection and parasite clearance [41–43].

One of our first approaches for antigen peptide backbone modification consisted in introducing topochemical elements into the selected antigen primary structure, which consisted in two key features, first the amino acids chirality and second the peptide backbone space orientation. As represented in **Figure 6A**, the N-terminus low polymorphic region of the *Pf*MSP1$^{42\text{-}61}$ native sequence (GYSLFQKEKMVLNEGTSGTA) was the base for evaluating the above described considerations. Thus, chirality impact on immunogenic properties was tested by introducing local and global *L*- and *D*-amino acid substitutions, and the peptide backbone orientation influence was assessed by reversing the primary structure but upholding its native composition. Therefore, a set of peptido-mimetic analogues were designed and synthesized and subsequently tested. As observed, the experimental group consisted in the *Pf*MSP1$^{42\text{-}61}$ native

sequence, entirely made of *L*-amino acids; its chiral D-analogue which preserved the sequence and was built with *D*-amino acids (represented in lowercase letters); another so-named ***Retro*** analogue sequence, which was constructed with *D*-amino acids and reversing the sequence orientation and an analogue called ***Retro-inverso***, which was synthesized with *L*-amino acids and reversing the peptide sequence orientation. Also punctual or partially D-substitutions were include in the experimental group. CD profiles for the four molecules reflected interesting spectrophotometric properties, thus when comparing the native L-sequence (black line) with its D-enantiomer (red line), their CD profiles behave as specular images of one another as displayed in **Figure 6B**. Similarly, CD profile for the L-native sequence compared with that of the **Retro-inverso** analogue (green line), behave as mirror images of each other bearing in mind that both were made with only L-amino acids, but having opposite peptide backbone orientation.

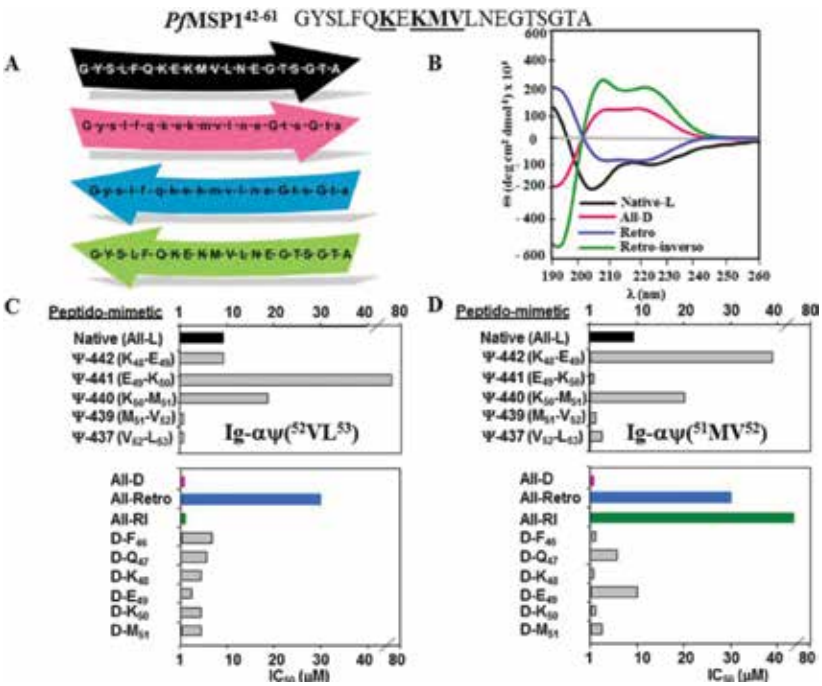

Figure 6. Chiral and topochemical peptido-mimetic designing. **(A)** Molecular peptido-mimetic design based on the N-terminus *Pf*MSP1$^{42\text{-}61}$ peptide. The native sequence is represented in capital letters for L-amino acids, D-enantiomer represented by lowercase letters purple highlighted, Retro-sequence is constituted by *D*-amino acids in lowercase letters blue highlighted and the so-named Retro-inverso peptidomimetic is confirmed by *L*-amino acids green highlighted. **(B)** Circular dichroism secondary structure patterns for *Pf*MSP1$^{42\text{-}61}$ peptido-mimetics. Used color code was black line for the native sequence, purple for its D-enantiomer, blue line for the Retro-analogue and green line for the Retro-inverso peptido-mimetic. Competition ELISA was used for the *Pf*MSP1$^{42\text{-}61}$ peptide mapping with monoclonal antibodies coded Ig-αψ-437(^{52}V-L^{53}) and Ig-αψ-439(^{51}M-V^{52}) as shown in **(C)** and **(D)**, respectively. IC50 μM values represent the average of data obtained in triplicate.

CD patterns for the D-enantiomer and the ***Retro-inverso*** analogue had a close relationship regarding the all-***L*** native sequence. On the other hand, CD profiles for the all-D-enantiomer

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(red line) and the ***Retro***-analogue (blue line) behaved as specular images of each other, keeping in mind that these molecules are made of only ***D***-amino acids, having the second a reversed backbone regarding the first. Interestingly, CD patterns of both, the L-***native*** sequence and ***Retro***-analogue resemble each other but are opposite to the CD profile of the D-enantiomer. Therefore, a partial conclusion emerged from these findings, independently of the amino acid composition; backbone orientation seemed to play a key role for the 3D structure properties of the antigen molecule.

In order to test the unique recognition of an antibody stimulated by a reduced amide peptido-mimetic in which the oxygen atom of the carbonyl group (–R–CO–NH–R'–) of a relevant peptide-bond was replaced with two hydrogen atoms to lead an analogue being the reduced form of it and herein named reduced amide peptido-mimetic ψ(–R–CH_2–NH–R'–). Thus, two monoclonal immunoglobulins (mAb) were produced, one directed to *Pf*MSP1$^{42\text{-}61}$ modified between the -^{52}V-L^{53}- and another directed to the -^{51}M-V^{52}- amino acid pairs, respectively, which herein are named as **Ig*α*-ψ-VL$^{52\text{-}53}$** and **Ig*α*-ψ-MV$^{51\text{-}52}$**. Both mAbs possess inhibitory capacities in both *in vitro* and *in vivo* malaria infections by *P. falciparum* as well as *P. yoelii* and *P. berghei* as elsewhere published [42]. Also our experiments allowed to a fine mapping of the MSP-1 N-terminus portion using monoclonal and polyclonal antibodies induced by reduced amide peptido-mimetics of the *Pf*MSP1$^{42\text{-}61}$ peptide and so we identified the antigen epitope whose amino acid sequence is ^{51}MVLNEGTSGTA^{61} as elsewhere reported [44].

Therefore, a whole set of *Pf*MSP1$^{42\text{-}61}$ modified analogues were used in competition assays for their ability to bind these mAbs. As observed in **Figure 6C** and **D**, the non-modified native sequence (all-L) bound at a 10 μg/mL while their inducer modified peptido-mimetics did it at a 1 μg/mL concentration. The reduced amide modification between 49E-K50 residues was an excellent competitor for binding to the **Ig*α*-ψ-MV$^{51\text{-}52}$**. Interestingly, the ***All***-D-enantiomer and ***Retro-inverso*** form of the sequence behaved as strong competitors for the **Ig*α*-ψ-VL$^{52\text{-}53}$** binding, both of them are built with ***D***-amino acids. In a similar way, most partially made D-substitutions were strong binders to both tested antibodies. Thus, both chirality and back-bone orientation become critical properties for antigen-antibody recognition, considering that natural features have to be resembled in artificially-modified immunogens, as well as preserving the molecule structure topology but most relevant it is an appropriate side-chain space orientation, which will be crucial for binding and functional effects.

In another set of experiments conducted based on the *C*-terminus low polymorphic portion the MSP1 antigen, the *Pf*MSP1$^{1282\text{-}1301}$ peptide whose primary structure EVLYLKPLAG-VYRSLKKQLE was the basis for protection capacity assays against malaria in Aotus monkeys immunized with reduced amide peptido-mimetics and partially made D-mutations. Animals were treated in agreement with Colombian and environmental regulations, and those individuals that developed malaria infection after being experimentally challenged with controlled doses of *P. falciparum* were subjected to medication to ensure their health conditions.

As observed in **Figure 7**, a few number of animals vaccinated have controlled the *Plasmodium* parasitemia levels, especially two out of ten vaccinated with the reduced amide ψ-^{7}P-L^{8} peptido-mimetic and four out of eight with a partial D-substitution in K^{6} of the *Pf*MSP-1$^{1282\text{-}1301}$ sequence.

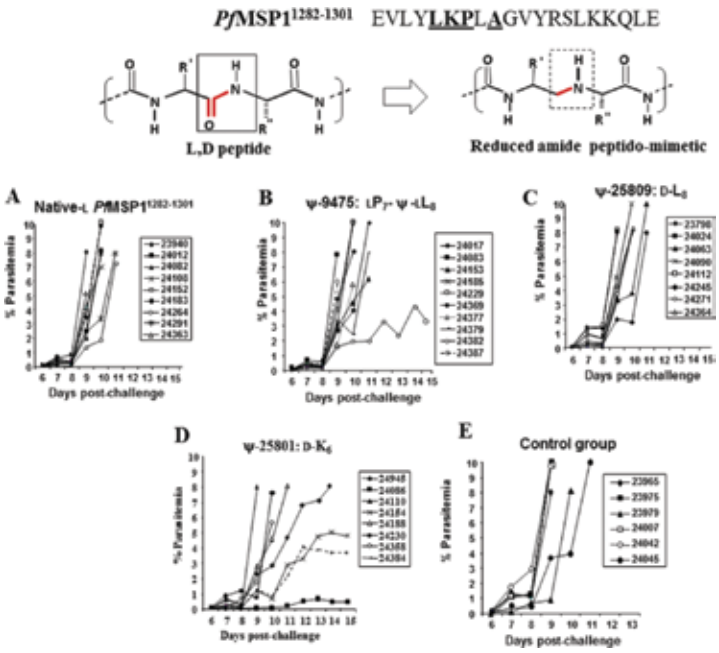

Figure 7. Protection capacity of *Pf*MSP-1/1282-1301 peptido-mimetics. **(A)** Aotus monkeys immunized with the *Pf*MSP-1$^{1282-1301}$ native sequence. **(B)** Animal group immunized with the ψ-9475 (^{7}P-L^{8}) reduced amide peptido-mimetic analogue. **(C)** Animals immunized with the partially substituted ***D***-L^{8} analogue. **(D)** Group of animals immunized with the partially substituted ***D***-K^{6} analogue. **(E)** Aotus monkeys immunized with saline solution as the placebo control group.

On the contrary, animals of the placebo-control and those vaccinated with the native sequence became faster infected and did not control the *Plasmodium* parasitemia. Therefore, the evidence supported the relevance of chiral-space occupancy as well as topochemical modifications as being important elements to be considered for malaria vaccine designing.

In order to be consisting with our proposed molecular models, we decided to focus our attention in another relevant *Plasmodium* antigen, the so-named *Pf*MSP-2 surface antigen. Specifically, we have designed and synthesized some reduced amide peptido-mimetics based on the N-terminus *Pf*MSP2$^{21-40}$ peptide whose amino acid sequence is KNESKYSNTFIN-NAYNMSIR. Thus, two peptido-mimetic analogues coded ψ-128 and ψ-130 which were modified between the ^{30}F-I^{31} and ^{31}I-N^{32} amino acid pairs, respectively, were obtained and characterized. Our experiments for a fine *Pf*MSP2$^{21-40}$ sequence mapping with monoclonal antibodies directed to both modified motifs have revealed a functional epitope whose exact location was ^{25}KYSNTFIN^{32} as previously published [45].

As observed in **Figure 8**, the reactivity patterns of both antibodies by western blot analyses lead to identified native *Pf*MSP2 protein fragments stained at 30.54, 34.21 and 37.90 kDa on a *P. falciparum* FCB-2 membrane protein lysate. Faster polypeptide fragments of *Pf*MSP2 on the SDS-PAGE, could be associated to this antigen cleavage during blood-stage parasite maturation to mature schizonts, previously to merozoite releasing (**Figure 8A**).

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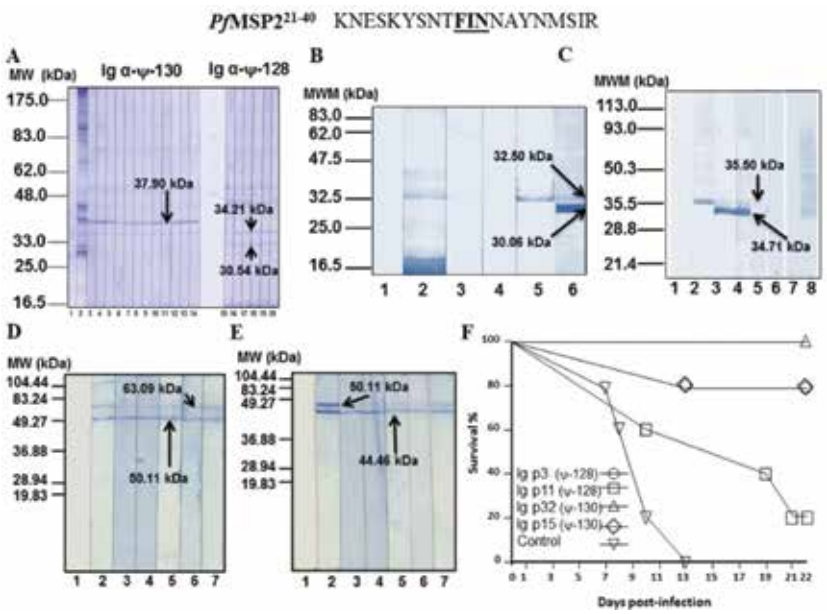

Figure 8. Reactivity and functional properties of *Pf*MSP2[21-40] peptido-mimetics. **(A)** *Plasmodium falciparum* FCB-2 membrane protein lysed resolved on a 7.5–15% SDS-PAGE gradient system. **(B)** Recombinant MSP2-$(His)_6$ protein treated with the Ig-αψ-128 (^{30}F-I^{31}) monoclonal antibody. **(C)** Recombinant MSP2-$(His)_6$ protein treated with the Ig-αψ-130 (^{31}I-N^{32}) monoclonal antibody. **(D)** Surface membrane proteins from blood stages of *P. berghei* treated with the Ig-αψ-128 and Ig-αψ-130 monoclonal antibodies. **(E)** Surface membrane proteins from blood stages of *P. yoelii* treated with the Ig-αψ-128 and Ig-αψ-130 monoclonal antibodies. **(F)** BALB/c mice infected with lethal doses of rodent malaria treated by passive transference of Ig-αψ-128 and Ig-αψ-130 monoclonal antibodies.

In order to verify the antibody reactivity, an *Escherichia coli* recombinantly MSP2 expressed fragment which contained part of the *Pf*MSP2 N-terminus sequence was employed. Hence, the Ig-αψ-128 antibody detected lysate produced bands at 30.06 and 36.50 kDa and the Ig-αψ-130 antibody recognize bands at 34.71 and 35.50 kDa, all polypeptide bands contained the MSP2 recombinantly expressed fragment as compared with the control raw, as shown in **Figure 8B, C** respectively.

Similarly, a lysate composed by membrane proteins from blood stages of *P. berghei* and *P. yoelii* were analyzed for these mAbs recognition. Therefore both Ig-αψ-128 and Ig-αψ-130 antibodies detected bands at 50.11 and 63.09 kDa mobilities for *P. berghei* ANKA and 44.46 and 50.11 kDa for the *P. yoelii* 17XL strain, respectively, as observed in **Figure 8D** and **E**. Besides, functional *in vivo* activity of these antibodies was tested by passive transferring experiments of both into *P. berghei* and *P. yoelii* infected BALB/c mice groups. Most animals survived to the lethal challenging with *Plasmodium* strains and efficiently have controlled parasitemia levels due to the antibody therapeutic activity as it was published and observed in **Figure 8F** [45]. This set of experiments revealed the importance of performing peptide-backbone strategic modifications by introducing non-natural elements into the immunogen primary structure, but its amino acid sequence identity has to be preserved to avoid any non-specific or non-desirable cross-reactive effects.

To obtain a complete landscape of this novel scenario, further scopes of the strategy of obtaining next generations of malaria vaccine candidates based on introducing non-natural elements into immunogens, trials performed in selected antigens of other *Plasmodium* stages, such as those called pre-erythrocytic as well as sexual forms on macro and micro-gamete particles have to be conducted. Hence, the circumsporozoite surface protein (CSP) expressed on pre-erythrocyte forms offers a classical interesting target for vaccine candidate development.

As reported before, a class-I restricted *Pb*CSP$^{252\text{-}260}$ epitope was identified in the CSP primary structure of *Plasmodium berghei*, a rodent malaria specie [46–49]. Thus, in order to test our hypothesis, we conducted experiments by introducing reduced amide peptide-bond isosteres in a systematic fashion and their subsequent evaluation regarding antibody stimulation and their reactivity was performed. This epitope whose amino acid sequence is SYIPSAEKI was the basis for the molecular designing. Thus, a set of peptido-mimetic analogues were synthesized and characterized. As shown in **Figure 9B** and **C**, antibodies induced by the *Pb*CSP$^{252\text{-}260}$ native sequence have some reactivity for both sporozoite and gametocytes as analyzed by indirect immunofluorescence assays (IFA) experiments while a stronger reactivity of the modified ^{257}A-E^{258} amino acid pair of *Pb*CSP$^{257\text{-}258}$ peptido-mimetic-induced antibodies, become evident regarding both sporozoites and gametocytes as observed in **Figure 9E** and **F**. Antibodies of pre-immune sera did not show any reactivity as seen in **Figure 9A** and **D**.

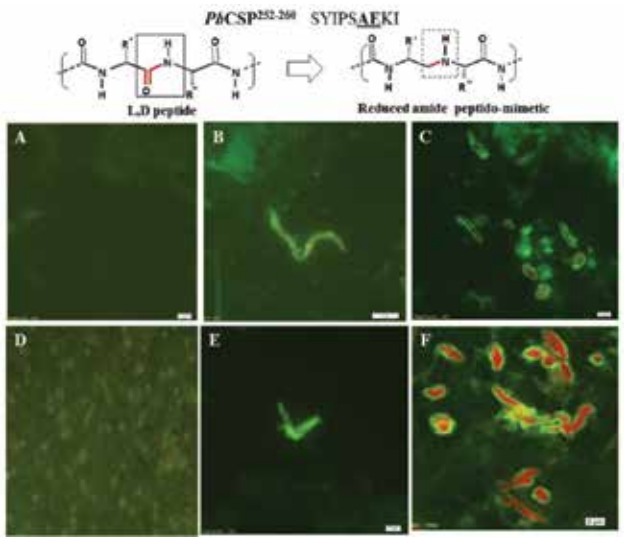

Figure 9. Exploring immunological properties of the restricted class-I *Pb*CSP$^{252\text{-}260}$ epitope by indirect immunofluorescence assays (IFA). **(A)** Pre-immune serum of mouse 2. **(B)** *Plasmodium falciparum* sporozoites detected by antibodies induced by the *Pb*CSP$^{252\text{-}260}$ native peptide post-third boost (mouse 2), image captured at 2 μm. **(C)** Detection of *Plasmodium falciparum* (NF54 strain) gametocytes by antibodies induced by the *Pf*CSP$^{252\text{-}260}$ native sequence post-third boost (mouse 2), and image recorded at 2 μm. **(D)** Reactivity of pre-immune serum of mouse 23. **(E)** Detection of *Plasmodium falciparum* sporozoites by antibodies to the *Pb*CSP (A–E) peptido-mimetic obtained post-third boost (mouse 23), image recorded at 1 μm. **(F)** Detection of *Plasmodium falciparum* (NF54 strain) gametocytes by antibodies directed to the *Pb*CSP (A–E) peptido-mimetic (mouse 23), and image captured at 2 μm.

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The proposed hypothesis has been confirmed by challenging it in different molecular scenarios, all based on analysis of different antigens derived from different *Plasmodium* species and stages and proved in *in vivo* and *in vitro* assays. Thus, an emerging conclusion states the importance of a careful and strategic molecular designing of potential malaria vaccine candidates which consider the antigen global structure modulation but preserving their specific fingerprint represented by their amino acid sequence.

Aimed to understand a possible structure-immunological activity relationship, a subsequent set of nuclear magnetic resonance NMR and molecular dynamic *in silico* experiments lead us to compare in all cases, both the native antigen, as well as their modified derivatives regarding their 3D structure properties. Thus, generated data are presented in **Figure 10**, in which either native antigen backbone conformation is overlapped with that of its functional representative peptido-mimetics or polypeptide conformations of homologue proteins in two *Plasmodium* species are compared.

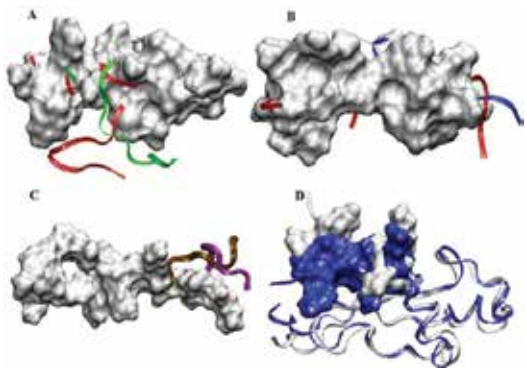

Figure 10. Structure-immunological activity relationship. Overlapped backbone of native and modified peptido-mimetic analogues were organized as follow. **(A)** Accessible solvent surface of the *Pf*MSP1$^{42\text{-}61}$ native sequence in white and ψ-437 in red ribbons and ψ-439 in blue ribbons. **(B)** Accessible solvent surface of the *Pf*MSP1$^{1282\text{-}1301}$ native sequence, ψ-9473 in blue ribbons and ψ-9475 in red ribbons. **(C)** Accessible solvent surface of the *Pf*MSP2$^{21\text{-}40}$ native sequence, ψ-128 in purple ribbons and ψ-130 in ochre ribbons. **(D)** Accessible solvent surfaces in white for *Pf*CSP and blue for *Pb*CSP. The ^{308}SYIPSAEKI^{316} class-I restricted epitope is highlighted.

As observed in **Figure 10A**, overlapped 3D conformations of the *N*-terminus *Pf*MSP1$^{42\text{-}61}$ peptide whose solvent accessible surface depicted in white, and two of its peptido-mimetics those coded as ψ-437 (^{52}V-L^{53}) and ψ-439 (^{51}M-V^{52}) represented by red and green ribbons, revealed deep structure differences between them. A highly compact α-helix structure present in the native sequence became flexible in its two peptido-mimetic analogues due to a single peptide bond modification, in which the oxygen atom of the –CO–NH– amide function of a specific peptide-bond, was replaced with two hydrogen atoms so leading –CH_2–NH– surrogates.

Similarly, backbone of the low polymorphic *C*-terminus of the same *Pf*MSP1 antigen specifically the *Pf*MSP-1$^{1282\text{-}1301}$ peptide fragment (solvent accessible surface in white) was overlapped with its two derive ψ-9473 (^{6}K-P^{7}) and ψ-9475 (^{7}P-L^{8}) peptido-mimetics (red and blue ribbons)

[50]. As in the first analyzed case, a highly compact α-helix structure present in the native sequence became flexible in its two peptido-mimetic analogues due to single peptide bond modifications which surely have introduced new freedom degrees to the molecule, as seen in **Figure 10B**.

On the other hand, backbone structural analyses for the *Pf*MSP2$^{21\text{-}40}$ regarding its two ψ-128 (^{30}F-I^{31}) and ψ-130 (^{31}I-N^{32}) reduced amide peptido-mimetics, revealed a close-related behavior regarding the couple of the above discussed examples for a different *Plasmodium* protein, the *Pf*MSP1. **Figure 10C**, displays the β-stranded conformation of the native sequence (solvent accessible surface depicted in white) regarding the more flexible conformations of both of its peptido-mimetics (represented by purple and ochre ribbons).

An interesting observation become evident when backbone of two homologue proteins are overlapped regarding a class-I epitope region, as it was the case of the *Pf*CSP and *Pb*CSP, as shown in **Figure 10D**; consisted in that those overlapped polypeptide conformations, suggest an intermediate molecular state among them, which could be represented by a peptido-mimetic structure probe. Thus, the peptido-mimetic coded *Pb*CSP$^{252\text{-}260}$ which represents a peptide-bond surrogate located between the ^{257}A-E^{258} amino acid pair, thus this strategic peptide-bond replacement could be responsibly of the stimulated cross-reactive antibodies.

Further experiments in this pursuit will explore hypothesis on *in vivo* protection against malaria regarding CSP peptido-mimetics and will be conducted in order to assess the functional inhibitory activity of peptido-mimetics and their antibodies on malaria-infected mice through *Anopheles albimanus* mosquito bites.

The family of the herein presented structural modified compounds constitute molecular tools to be considered for new generations of functional protective vaccines against malaria, as such, future vaccine candidates could be based on this knowledge and outstanding findings.

Acknowledgements

As the author of this work, I am indebted to Prof. Manuel Elkin Patarroyo for his invaluable contribution to my personal view on the malaria vaccine field. Special thanks to the Colombian Science Technology and Innovation Department (Colciencias) (Grant No. 212456934488).

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http://dx.doi.org/10.5772/65251

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Chapter 16

Pre-Erythrocytic Vaccine Candidates in Malaria

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65592

Abstract

A vaccine providing sterile immunity against malaria has been shown to be possible with antigens from the pre-erythrocytic stages of malaria. Therefore, it is reasonable to focus vaccine development efforts on the pre-erythrocytic stages, consisting of both sporozoites and liver stage parasites, where it is expected that sterile immunity against the parasite can be elicited to block the development of blood stage infection, clinical disease, and resulting parasite transmission. Accordingly, we will review the preclinical and clinical studies of malaria pre-erythrocytic efforts as well as highlight the advances, trends, and roadblocks encountered in these efforts.

Keywords: *Plasmodium falciparum*, malaria, vaccine, clinical trial, CSP, TRAP, CelTOS, LSA, EP1300, STARP, EXP-1, immune response, humoral, cellular, CD4+, CD8+

1. Introduction

Immunity to malaria correlates with age and develops only after years of repeated exposure to bites from infected mosquitoes [1, 2]. Most adolescents in malaria-endemic regions have developed a level of protective immunity that provides resistance to clinical disease but does not fully eliminate infection [1]. This lack of sterile immunity does not prevent further spread of the disease, as some level of parasitemia persists in these individuals. A complete understanding of how the malaria parasite avoids clearance remains unknown. However, mechanisms such as extreme allelic variation, strain diversity, as well as the unique complexity of the malaria parasite life cycle are undoubtedly involved. In this regard, identification of the conserved, critical epitopes of the parasite that are directly associated with eliciting

protection may provide the best viable target node for vaccine development. In addition, recent data from mouse models of malaria suggest that T cell exhaustion significantly impairs development of effective immune responses and contributes to chronic malaria infection [3–5]. Notwithstanding the fleeting immunity that individuals receive from frequent infection with the parasite, it suggests that there are targets for the immune system to attack.

Importantly, exposure to attenuated sporozoites that can infect hepatocytes but do not develop into the blood-phase of malaria can induce immunity that eliminates parasitic infection (i.e., sterile immunity). This has been demonstrated by conducting immunizations with irradiated sporozoites both in mouse models of malaria [6] as well as in humans with different strains of *Plasmodium falciparum* [7–10]. Similarly, induction of sterile immunity can occur in individuals undergoing chloroquine or mefloquine chemoprophylaxis. The resulting "chemically attenuated" *Plasmodium falciparum* sporozoites (CPS-CQ or CPS-MQ), which undergo full liver stage development but do not develop to clinical blood-stage infection [11, 12], can induce sterile immunity [12–15]. Of note is that sterile protection may not fully develop when sporozoites do not progress at least partially through liver stage development, potentially providing stage transcendent protection [16, 17]. These discoveries demonstrate that a vaccine providing sterile immunity against malaria is possible with antigens from the pre-erythrocytic stages of malaria. Therefore, it is reasonable to focus vaccine development efforts on the pre-erythrocytic stages, consisting of both sporozoites and liver stage parasites, where it is expected that sterile immunity against the parasite can be elicited to block the development of blood stage infection, clinical disease, and resulting parasite transmission.

1.1. Correlates of immunity

The correlates of immunity for sterile protection to malaria are not well defined [18, 19]. In both mice and humans immunized with irradiated sporozoites, sterile protective immunity is believed to be directed to both sporozoites and liver stages of malaria [1, 18]. Antibodies to sporozoites can opsonize sporozoites or neutralize invasion into hepatocytes, and potentially act synergistically with T cells to confer sterile immunity [20]. Additionally, several monoclonal antibodies against the major sporozoite surface antigen, the circumsporozoite protein (CSP), are capable of mediating protection upon passive transfer into animals [21].

Regarding cellular response, studies in mice involving depletion of CD4+ and CD8+ T cells indicate a T cell response is essential for protective immunity [22–24]. In mice immunized with irradiated sporozoites, IFN-γ produced by CD4+ and CD8+ T cells is believed to be the main antiparasitic effector produced by the immune response [18, 23, 25, 26]. IFN-γ may activate effector cells, such as natural killer cells and macrophages that can target and kill parasite-infected liver cells. CD8+ CTLs that recognize *Plasmodium* antigens presented by major histocompatibility complex (MHC) class I molecules on parasite-infected liver cells also may target those cells for destruction [25, 27–29]. This has led to identifying epitopes and vaccination approaches against malaria that elicit CD8+ T cell-mediated IFN- γ secretion and development of CTLs [25, 28, 30]. However, it is important to note that in studies of mice with incapacitated CD8+ T cell function, CD4+ T cell-dependent sterile immunity can be elicited upon immunization with irradiated sporozoites [31]. Additionally, studies evaluating peptide

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vaccines in mice indicate that CD8+ T cells may not be necessary for sterile immunity but rather CD4+ T cells provide a critical role in immunity [18, 32–35]. Thus, in mice there is not a definitive marker for sterile immunity, and it is plausible that different immune responses may jointly contribute to protection.

In humans, immunization with irradiated sporozoites induces protective immunity consisting of both humoral and cell-mediated responses. Cell-mediated immunity comprises of a mixed population of CD4+ and CD8+ T cells producing IFN-γ and cytotoxic activity, both of which have been shown to possess antiliver stage activity [19, 25]. However, upregulation of CD8+ T cells does not always correlate with protective immunity in humans [10, 19, 25]. For example, immunity elicited in humans under chloroquine chemoprophylaxis with *Plasmodium falciparum* sporozoites is associated with CD4+ T cell production of IFN- γ, TNF, and IL-2 [12, 14], but not with upregulation of CD8+ T cells. Although the role of CD8+ T cells in mediating sterile protection in humans is still unclear, it appears that multiple factors including antibody, CD4+ and CD8+ T cells collectively contribute to overall protective immunity in humans.

1.1.1. Immunological challenges

Two genetic restrictions impact development of vaccines to malaria. The first genetic restriction is related to the extreme heterogeneity of the *Plasmodium* genome and resulting proteome, and existence of a large variety of different circulating strains (particularly for *P. falciparum*) that produce antigens that vary in sequence. As a result of this extreme heterogeneity, in many cases, efforts to identify antigens that can elicit protection against a broad range of malaria strains have been limited to the antigens that are the least variable. In this case, proteins selected for vaccine development are restricted to those relatively conserved across strains, which limits the available vaccine targets. The second restriction involves the population of HLA alleles on each person's cells that recognize these vaccine candidates and are involved in the induction of an immune response. Because of HLA polymorphism, individuals in a population will not generally recognize the same epitopes in an antigen. That is, a single individual in a given population will have HLAs that are capable of binding only a subset of the epitopes present in a vaccine. This means that a single epitope may only stimulate an effective immune response in a small percentage of any population, depending on the representation of the relevant HLA allele(s) in that population [28, 36–38]. The combined effect of these two restrictions is that an effective vaccine must provide many antigenic epitopes to ensure an effective immune response to the spectrum of circulating *Plasmodium* strains is elicited in a significant percentage of a population.

The need for a multiantigen vaccine has resulted in a number of studies identifying conserved epitopes in *Plasmodium* proteins that can stimulate CD4+ or CD8+ T cells from individuals either exposed to malaria or immunized with sporozoites [36–38]. The approach has been refined to identify promiscuous peptides capable of binding more than one type of HLA, resulting in antigens that can affect immune response in a larger percentage of the population. Incorporation of these epitopes into chimeric recombinant proteins for processing by immune cells to produce the peptides and stimulate T cells has been done [39, 40]. Examples of this approach are the multiepitope strings in ME-TRAP [41] and EP1300 (Section 4). Unfortunately,

in these constructs, the epitope strings produced low immune responses, even when other whole proteins in these vaccine compositions elicited a robust immune response [42–45].

Another confounding element to vaccine development, in general, is the elicitation of regulatory T cells (Tregs). It is understood that Tregs represent a "self-check" for providing immune tolerance to self and preventing the damaging effects of immune overreaction. For example, Tregs are involved in shutting down immune responses after they have successfully eliminated invading infectious agents, as well as their endogenous role of preventing autoimmunity [46]. However, some pathogens have been found to exploit development of Treg responses in order to evade elimination and persist in the human host. Tregs can also be induced by vaccination and some vaccine development efforts, such as those against tumors and chronic infection (e.g., tuberculosis), have included steps to overcome the constraints of such vaccination-induced Treg responses that result in suppression of immunity [47]. One somewhat successful approach pursued by biotechnology and pharmaceutical companies is development of adjuvant formulations, small molecules, and monoclonal antibodies to overcome the induction of and/or inhibit Treg function [48]. This area of study is also important regarding the development of malaria vaccines, as there is a growing set of data to support the conclusion that induction of Tregs during acute malaria infection limits the generation of immune memory and increases susceptibility to infection [49]. Of particular concern for malaria vaccine development efforts is that in the infant target population, functional Tregs are present in high numbers [49]. Therefore, tools to identify and remove Treg epitopes from malaria vaccines could be critical to the development of candidates that induce strong T cell responses.

1.1.2. Antigen discovery efforts

The understanding that infection with attenuated sporozoites can confer sterile immunity led to a new focus on discovery of liver-stage antigens [1]. Serological approaches to antigen discovery have been used in malaria research; however, sera from chronically infected individuals predominantly recognize the blood-phase of infection [50]. Therefore, discovery of liver-stages antigens has required methodologies that exploit recent discoveries of immunity targeting the liver-phase of infection. For example, immunological approaches using sera from individuals on antimalarial therapy (CPS-CQ or CPS-MQ) while in malaria-endemic regions were applied to screen libraries expressing *Plasmodium* peptides [51, 52]. Later, similar approaches were applied using blood from individuals immunized with irradiated sporozoites to evaluate expression libraries for reactivity with antibodies [53, 54], or T cell responses with peripheral blood mononuclear cells (PBMCs) [55]. These types of approaches continue to be a source of new antigens [56].

The availability of numerous genomic sequences from *Plasmodium* species has greatly facilitated such studies and made possible further large-scale genomic screening approaches that apply transcriptional analysis using isolated sporozoites with targeted comparisons to different phases in the *Plasmodium* lifecycle [57–61]. When applied to vaccine discovery, the genomic screens typically include a selection of proteins shown to react with antibodies or responsive T cells from individuals immune to *Plasmodium*. On a smaller scale, specific stages of the parasite's lifecycle have been evaluated using proteomic analysis [54, 62, 63]. These

efforts have been augmented by extensive cross-comparison of results to prioritize those antigens identified by more than one approach. Many of these antigens, which show a relatively conserved primary protein structure, are being evaluated and developed as components of subunit vaccines against malaria (Section 5).

1.1.3. Surrogate models of protection

One of the challenges in vaccine development is the need for an animal model that clearly correlates to immunity and efficacy in humans. Mice are frequently used to evaluate antigens for both the resulting immune response and efficacy to preventing infection. Both inbred and outbred strains have been evaluated. While inbred strains (e.g., BALB/c and C57BL/6) provide more consistent results, outbred strains (e.g., CD1 and ICR) are believed to better represent the diversity of the immune response that is encountered in humans due to the polymorphism of the MHC (HLA in human). As *Plasmodium* strains that infect humans do not infect rodents, mouse models of malaria either require (1) use of rodent malaria species (e.g., *P. berghei*, and *P. yoelii*) and immunization with the rodent malaria ortholog of the target antigen, which is generally divergent from the corresponding vaccine target from a *Plasmodium* species that infects humans (e.g., *P falciparum* and *P. vivax*); or (2) development of chimeric parasites where the rodent malaria ortholog of the target antigen has been replaced by the corresponding protein from a human malaria species. In some cases, orthologs of the vaccine targets do not exist in the rodent malaria species, making evaluation in animal models difficult (e.g., LSA-1 and SIAP2 [61], and SIAP2 [61, 64]). While chimeric rodent malaria strains that express the *P. falciparum* antigen have still been used in this type of scenario [33], the impact upon the physiology of the parasite, the host-pathogen interaction, and resulting response to vaccination is not clear. In rare cases, orthologs are sufficiently similar between the rodent and human malaria species to permit immunization using the human malaria antigen and challenge using a wild-type rodent malaria species [65]. While mouse models of malaria can be useful, immune response and level of protection to a target antigen can vary when different rodent malaria strains/species or chimeric models are used, making analysis difficult as it is not clear which of these datasets may be most applicable to or predictive of *P. falciparum* in humans [19, 28].

The use of nonhuman primate (NHP) models provides a more phylogenetically related model of human disease for both the host and parasite. However, cost and the regulations concerning research with NHPs have limited the use of these models. Even when used to evaluate immunity and safety of vaccines, NHPs are typically not challenged with sporozoites, so vaccine efficacy cannot be determined [66–68]. Further, interpretation of results using a NHP model must be balanced with the realization that old-world monkeys do not have the ortholog to the human HLA-C locus, which is one of the three classical human MHC class I loci [69]. In humans, HLA-A and HLA-B (the other two classical human MHC class I loci) exhibit extreme diversity through extensive allelic polymorphism in each class I gene [70]. Whereas in *Rhesus macaque* (the best characterized old-world monkey), there are very limited morphotypes for class I genes, but relative to humans there are more types of class I genes and extensive diversity of the combination of the genes resulting in a potential 10-fold greater diversity of proteins in NHPs [70, 71]. Therefore, the limited overlap of NHP and human MHC, with each species

containing unique MHC genes, and the much larger repertoire of MHCs in NHP makes it difficult to extrapolate results in NHP immune response to predict the response in humans. Thus, the immune response of NHPs to peptides developed for a human vaccine must be cautiously interpreted, and extrapolation of the results to define an appropriate dosage for use in humans may be problematic. Because of these limitations, many antigens have rapidly progressed to testing in humans with very limited efficacy testing in animals.

Lastly, it is noteworthy that several research groups have recently developed humanized mouse models that mimic human immune system for the purpose of testing human malaria vaccines. These humanized mice can mount both human T cell and antibody-mediated immunity in the context of HLA-restriction [72–74]. Other efforts have been made toward creating a mouse model having human liver [75–79]. These humanized liver mice can sustain development of human malaria parasites from sporozoites to blood stages.

2. Pre-erythrocytic vaccines based on CSP

2.1. Structure and antigenicity

One of the most studied of malaria proteins is the sporozoite surface antigen CSP, which forms a dense coat surrounding the parasite. This protein is involved in sporozoite motility and invasion where contact of the N-terminal region of CSP with hepatocytes triggers cleavage of the N-terminal region from the remainder of the protein [80–82]. This cleavage event is required for sporozoite infection of hepatocytes [80, 81, 83]. Apart from the central repeat region, CSP contains two regions of high conservation: Region 1 in the N-terminal third of the protein that includes the proteolytic cleavage site, and Region II in the C-terminal third of the protein; the latter has some hepatocyte binding activity [82, 84] and is located in a section of CSP enriched in T cell epitopes (**Figure 1**).

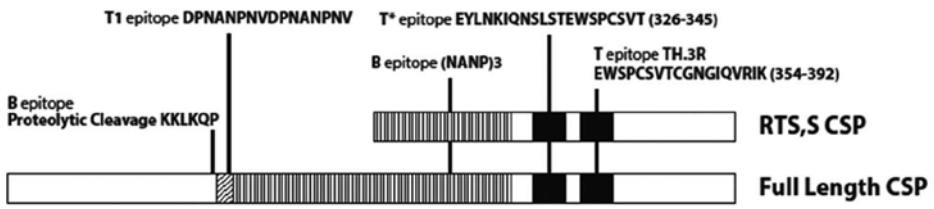

Figure 1. Schematic of full-length CSP identifying important epitopes.

Over 30 years ago, NANP repeats in the central repeat region of the protein were identified as the target of protective antibodies [85–88]. The T1 epitope, which is located in the *P. falciparum* CS minor repeat region, comprised of alternating NANPNVDP repeats, was originally identified by human CD4+ Th1-type T cells from a volunteer immunized with irradiated *P. falciparum* sporozoites [87]. Multiple CD4+ and CD8+ T cell epitopes have been identified in

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the C-terminal region of CSP, and these are recognized by murine and human cells. Several CD4+ T cell epitopes in this region, labeled as T*, UTC, or TH2 in various publications, include one or more epitopes termed "universal" as they are recognized by a broad spectrum of HLA class II molecules. The T* epitope is present in the C-terminus of CSP amino acids 326–345 and promotes both cytotoxic and helper CD4+ T cell responses. The T* epitope was identified using CD4+ T cell clones derived from volunteers immunized by multiple exposures to the bites of irradiated *P. falciparum* (NF54 strain) infected mosquitos [89]. The T* epitope spans part of a polymorphic region as well as a portion of the conserved Region II. Different CD4+ T cell clones selected from these irradiated sporozoites volunteers recognized truncated peptides (8–18 amino acids long), all of which contained the RII sequence [89]. Importantly, the T* epitope, unlike the T1 epitope, is presented *in vitro* by multiple DR alleles reflecting a broad MHC class II binding pattern, thus suggesting a "universal CD4+ T cell epitope" for malaria [89, 90].

It has been speculated that CSP-specific T cells are required to provide protection through help for antibody production and CTL function, as well as direct cytokine-mediated antiparasite activity [22, 91]. However, the regions of CSP containing immunodominant T cell epitopes are also highly polymorphic among circulating *P. falciparum* strains [92], which is problematic regarding their use in vaccines.

2.1.1. RTS,S (Mosquirix)

The most advanced malaria vaccine (RTS,S) is a virus-like particle (VLP) consisting of a fragment (central repeat and C-terminal regions, amino acids 207–395) of the CSP fused to a hepatitis B virus surface antigen (**Figure 1**). Phase 3 clinical trials of RTS,S/AS01 (commercially known as Mosquirix), a liposomal adjuvant formulation AS01 which contains monophosphoryl lipid A (MPLA) and QS-21, have provided evidence for high levels of antiCSP antibody correlating with reduced clinical malaria episodes [93]. Interestingly, individuals immunized with irradiated sporozoites or RTS,S generate antibodies to the repeat region, which represents the immunodominant region of the protein under these circumstances; however, adults in endemic regions naturally infected with the parasite, and thus partially immune from disease, have high levels of antibodies against the C-terminus and other nonrepeat regions [94]. In malaria nonexposed individuals immunized the with RTS,S vaccine, protection correlated with CD4+ and CD8+ T cells responses to the C-terminal region of CSP [95], while in other studies, sterile immunity was correlated with antirepeat antibodies [96]. In endemic areas, protection against clinical disease in RTS,S immunized infants also correlated with antiCSP antibodies [97]. Altogether, the protective efficacy of the CSP-based RTS,S/AS01 vaccine is a clear demonstration that a recombinant subunit vaccine containing a portion of the protein delivered on a heterologous VLP carrier can provide partial protection in humans. As 30–50% protection against clinical disease was obtained in RTS,S immunized infants and children in endemic areas, it is clear that there is room for improvement upon the RTS,S vaccine.

2.2. Vaccines containing CSP repeats

Whereas there are several multiantigen pre-erythrocytic vaccines that include CSP repeat units along with other CSP epitopes, the vaccines described in this section contain only CSP repeat

units or CSP repeat units combined with epitopes from other malaria antigens. One of the earliest vaccine trials with epitopes from CSP was a Phase I trial with controlled human malaria infection (CHMI) where 35 volunteers were immunized with a three-unit NANP repeat peptide $(NANP)_3$ conjugated to tetanus toxoid (TT) and adjuvanted with alum [98]. As the CSP repeats are a known B cell epitope, the three volunteers with the highest ELISA and immunofluorescence assay (IFA) titers were challenged with *P. falciparum* sporozoites (strain NF54); one of these volunteers was sterilely protected. A second trial with CHMI was conducted with this same vaccine where 202 volunteers were immunized and four volunteers with the highest titers were challenged; however, no sterile protection was seen in the challenged volunteers [99]. The results from these trials spurred development of vaccines containing minimal epitopes from CSP that contain CSP repeat units as well as the combination of CSP repeat units with epitopes from other malaria antigen vaccine targets.

One effort to explore combination of CSP repeat units with another malaria vaccine target includes development of a series of influenza virosome-based vaccines. However, virosome-based antigen developed contains a slight variation of the CSP repeat units, NPNA rather than NANP [100]. The resulting vaccine incorporating a constrained $(NPNA)_3$ repeat was termed PEV302. In a Phase I study, 16 volunteers received PEV302 and eight volunteers received a combination vaccine containing PEV302 as well as an influenza virosome containing a portion of domain III from AMA-1, termed PEV301 [101]. Sera collected from volunteers were used to assess ELISA and IFA titer to the constrained repeat peptide and *P. falciparum* (NF54) sporozoites, respectively. Of the 21 volunteers immunized with PEV302, 19 demonstrated titers $\geq 10^2$ after three immunizations [102]; however, a challenge was not conducted as part of this study. A second trial that included CHMI was conducted with these vaccines where the PEV301 and PEV302 epitopes were combined (known as PEV3A) and administered together to 12 volunteers [103]. In this same trial, 13 volunteers were co-immunized with PEV3A and ME-TRAP. Both groups were challenged; however, all volunteers developed parasitemia. The ELISA titers to UK-39 constrained repeat peptide and IFA titers *P. falciparum* (NF54) sporozoites elicited in both studies were similar with all individuals in the second study demonstrating titers $\geq 10^2$.

2.3. Vaccines containing minimal CSP epitopes

It has been suggested that malaria, along with other infectious diseases (e.g., HIV), has resisted classical vaccine strategies by utilizing a method of stimulating either strain-specific or incomplete, nonprotective immunity, which appears to have arisen from having co-evolved with humans for millions of years [104]. Further, studies with CSP in particular have suggested mechanisms other than allelic variation for parasite escape, even suggesting that the immune-dominant repeat region presents as an immunogenic "decoy" that elicits nonneutralizing antibody responses [105]. In general, the majority of vaccine developed strategies have targeted pathogens that display little antigenic variation, are highly conserved among isolates, and are readily inhibited by immune responses stimulated by native antigens, which is not the case for malaria. Furthermore, that only partially protective immune responses result after years of malaria exposure suggests that the parasites may be utilizing a "cloaking" strategy to hide

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in plain sight of the host immune system such as incorporation of human-like protein sequences to induce Treg expansion [106]. Indeed, for vaccine targets, it has been shown that the degree of cross-conservation of predicted epitopes with the human genome inversely correlates with their immunogenicity [107], and in the case of malaria, Tregs (via PD1 upregulation) are responsible for a low frequency of precursor T cells [5]. Therefore, utilizing a minimal, well-designed epitope set for one or more targeted antigens may provide the most effective means to circumvent the parasites' avoidance mechanisms.

Efforts to combine the CSP repeat unit with other CSP epitopes include development of a series of synthesized peptide constructs containing $(NANP)_3$ (also known as B), the T1 epitope $(NANPNVDP)_2$, and in later studies, the T* epitope (**Figure 1**). A Phase I trial of a synthetic multiple antigenic peptide (MAP) immunogen, which includes four linked T1B peptides adjuvanted in alum with or without QS21, found that better ELISA and IFA titers were achieved with QS21 formulations [108]. This study also demonstrated that recognition of the T1 epitope by T cells is highly MHC-restricted with T cell responses limited to high responder MHC class II genotypes (present in only 25–35% of the population). None of the volunteers in this study were challenged. To address limitations of the T1 epitope, the T* epitope (which is recognized in the context of many human MHC class II molecules) was included in a branched synthetic peptide immunogen containing four linked T1BT* peptides as well as a linked Pam3Cys as endogenous adjuvant [109]. In a small Phase I trial of the tetra-branched T1BT* peptide in 10 volunteers of diverse HLA types, the majority of the volunteers (8/10) seroconverted following the first dose and reached peak antirepeat antibody titers of 10^3–10^4 following three immunizations [110]. The immunized volunteers developed T*-specific Th1- type CD4+ T cell responses. CD4+ T cell clones derived from PBMC up to 10 months after peptide immunization recognized the T* epitope in the context of multiple HLA DR and DQ molecules and secreted high levels of IFN-γ and variable levels of TNF-α [111]. The immunized volunteers in this study were not challenged. Note that up to 1 mg of immunogen was safely administered per dose in these clinical studies.

Additional clinical trials have investigated novel delivery platforms and adjuvants to enhance immunogenicity of CSP minimal T and B cell epitopes. A VLP based on the hepatitis B virus core antigen, ICC 1132, was engineered to express the T1, B $(NANP)_3$, and T* epitopes [112]. A total of four clinical studies were conducted with this antigen. Phase I trials using alum as adjuvant elicited suboptimal antibody and cellular responses [113, 114]. Use of a more potent water-in-oil emulsion-based adjuvant, Montanide ISA 720, enhanced not only immunogenicity, but also reactogenicity in a NHP model [115]. Two clinical studies were conducted with ICC 1132 formulated in Montanide ISA 720; however, due to the potential for increased reactogenicity, only a single administration was given. In the first study with ICC 1132 in ISA 720, escalating doses of antigen (from 20 to 50 μg) were administered [116]. Both ELISA and IFA titers with a single dose of ICC 1132 in ISA 720 were improved as compared to those achieved with three doses of ICC 1132 in alum. A second clinical study was conducted where a single 50μg dose of ICC 132 in ISA 720 was administered to 11 volunteers that were subsequently challenged; however, none of the volunteers were protected [117]. A summary of Phase I clinical studies, with CSP vaccine candidates, is provided in **Table 1**.

Category	RTS,S				Pf CS 282–383	NANP-TT	(T1B)4	(T1BT*)4	ICC-1132			
Epitopes	NANP, aa 302–395 (207–395)				NANP, aa 302–383 (282–383)	(NANP)3	NANP, NVDP (T1)	NANP, NVDP (T1), (T*) aa 326–345	NANP, NVDP (T1), (T*) aa 326–345			
Carrier/platform	HBsAg				None	TT fusion	MAP	Polyoxime	HBcAg (truncated)			
Formulation/adjuvants	AS02	AS03	AS04	Alum	ISA 720	Alum	Alum +/− QS21	Pam3Cys	Alum, saline	Alum	ISA 720	ISA 720
Route	IM	IM	IM	IM	IM	IM	SC	SC	IM	IM	IM	IM
# Administrations	3 1–3	3	3 3	3	3	3	3	3	2–3	3	1	1
Dose	50μg 10μg, 25μg, 50μg	50μg	50μg	50μg	≥100μg	20μg, 50μg, 100μg, 160μg	500μg, 1000μg	1000μg	20μg, 50μg	20μg, 50μg	5μg, 20μg, 50μg	50μg
IFA: % titer >10^2 [group GMT]	100% [6400] 100% [3200–10500]	100% [4755]	75% [654] 100% [ND]	50% [ND]	100% [2364–2810]	23% [ND]	25–67% (+QS21) [5511]	70% [216]	25–63% [57–226]	33–71% [ND]	38–100% [ND]	ND
ELISA: % Seroconv. (titer >10^2), coat Ag	100%, NANP 100%, NANP	100%, NANP	100%, NANP 100%, NANP	100%, NANP	100%, CS 282–383	33–57%, NANP	25–86% (+QS21), spz	100%, (T1BT*)4	50–75%, (T1B)4	40–75%, (T1B)4	63–100%, (T1B)4	100%, NANP
Cellular proliferation	ND 50–86%	ND	ND 10%	0%	63% (ISA)	0%	86% (+QS21)	60%	47–75%	ND	50%	ND
Protection (# protected/# challenged)	86% (6/7) 50μg × 3 30% (3/10) 50μg × 1 50% (7/14) 50μg × 2 50% (3/6) 50μg ×3 57% (4/7) 25μg × 3 25% (1/4) 10 μg × 3	29% (2/7)	13% (1/8) 25% (2/8)	0% (0/6)	ND	33% (1/3)	ND	ND	ND	ND	ND	0% (0/11)
References	[121] [124]	[121]	[121] [122]	[122]	[119]	[98]	[123] [90]	[110] [90]	[113] [112]	[114]	[116]	[117]

Percentages indicate percent responders for IFA, ELISA, and cellular proliferation; ND, not determined; GMT, geometric mean titer.

Table 1. Summary of clinical trials of CSP vaccine candidates (including RTS,S CHMI studies).

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2.4. Long synthetic CSP peptide vaccines

In addition to vaccine studies using minimal T and B cell epitopes, clinical studies have investigated immunogenicity of long synthetic peptides containing the entire N- or C-terminus of *P. falciparum* CSP [118]. Of interest is the Pf CSP 282-383 study where a long synthetic peptide including C-terminus amino acids 282–383 from CSP (PfCS102) was synthesized and administered to volunteers as a formulation in either alum or Montanide ISA 720 [119]. Elevated ELISA and IFA titers were seen with ISA 720 formulation as compared to alum. In a second clinical trial, PfCS102 formulated in ISA 720 or AS02A elicited IFN-γ secreting CD8+ T cells specific to malaria in some individuals [120]. Tenfold higher antibody and cellular responses were obtained with the AS02 adjuvant formulation. However, no volunteers were challenged in these studies. Note that synthesized peptide doses of ≥100μg were administered in this study.

2.5. Preclinical pipeline of novel CSP-based vaccines

Additional preclinical studies of VLP have utilized a woodchuck hepatitis core antigen engineered to express CSP repeats in the loop region and multiple universal T cell epitopes at the C-terminus, termed Mal-78-3T VLP [125]. Mice immunized intraperitoneally (i.p.) with Mal-78-3T VLP, formulated in alum/QS21 or ISA 720 adjuvant, developed high levels of antirepeat antibodies. Sterile immunity was obtained in Mal-78-3T immunized mice of three different strains following challenge by transgenic sporozoites expressing *P. falciparum* CSP repeats.

The minimal T and B cell epitopes have also been tested as a recombinant protein using a TLR5 agonist, flagellin, as adjuvant in murine studies [126, 127]. Chimeric flagellin protein containing multiple T1BT* modules, or nearly full-length *P. falciparum* CSP, elicited high levels of antirepeat antibody. The chimeric protein was immunogenic without addition of exogenous adjuvant when delivered subcutaneously or as a needle-free intranasal vaccine.

In addition to the TLR 5 agonist, a TLR-7 agonist, imiquimod, was also found to be a potent adjuvant for vaccines containing minimal T and B cell epitopes. Immunization of mice with the linear T1BT* peptide subcutaneously followed by application of topical TLR-7 agonist imiquimod induced protective antibody-mediated immunity as well as splenic CD4+ T cell responses [128]. Recent Phase I/II studies have demonstrated that topical imiquimod can also enhance immunogenicity and protective efficacy of a seasonal flu vaccine [129, 130], supporting the use of the murine model for identification of promising TLR agonist adjuvants for human trials.

Of interest are murine studies demonstrating that micro- and nanoparticle vaccines containing minimal T and B cell epitopes were immunogenic without the addition of exogenous adjuvants. Layer by Layer (LbL) microcapsules were constructed by sequential layering of positively charged poly-l-lysine and negatively charged poly-L-glutamic acid with derivatized peptides containing T1BT* epitopes added to the final layer [131]. Mice immunized with LbL microparticles in PBS elicited sporozoite neutralizing antibodies that blocked *in vitro* invasion of transgenic *P. berghei* sporozoites expressing *P. falciparum* CSP repeats into human hepatoma

cells and reduced parasite liver burden following challenge by exposure to the bites of mosquitoes infected with the transgenic rodent parasites. Protection was comparable to levels obtained following immunization with T1BT* peptide in Freund's adjuvant.

Self-assembling proteins that form nanoparticles (SAPNs) have also been examined in murine studies [132, 133]. The SAPN is comprised of 60 monomers of a recombinant linear protein containing trimeric and pentameric coiled-coil domains separated by a flexible linker with T and B cell epitopes expressed at N- or C-terminus. Following expression in *Escherichia coli*, monomers self-assemble to form spherical SAPN of ~40 nm in diameter. A *P. falciparum* SAPN was constructed comprised of monomer containing four copies of the NANP repeats at the C-terminus and three previously identified *P. falciparum* CSP CD8+ T cell epitopes (KPKDELDY, MPNDPNRNV, and YLNKIQNSL) at the N-terminus, along with a designed pan-DR binding T cell epitope termed PADRE. All strains of mice immunized with two or three doses of SAPN developed high ELISA titers that persisted over 52 weeks and CD8+ T central memory cells secreting IL-2 and IFN-γ. Mice challenged by i.v. injection of transgenic rodent parasites expressing full length *P. falciparum* CSP protein showed 90–100% protection, with 50% of SAPN immunized mice still protected against sporozoite challenge at week 52. Protective immunity was mediated by both sporozoite neutralizing antirepeat antibodies and CD8+ T cells.

One notable target absent in most CSP-based constructs is the N-terminus of CSP, which contains both T and B cell epitopes as well as the highly conserved Region I (**Figure 2**). Further, within Region I, resides the proteolytic cleavage site and a ligand-binding domain which have been shown to be key requirements for sporozoite infection of hepatocytes [80, 81, 83]. Antibodies raised to this region have been shown to confer 90% protection in animal passive transfer studies [134]. Therefore, efforts are now being pursued to identify and incorporate this epitope into some of the strategies described in this section.

Figure 2. Diagram of LSA-1 (adapted from [164]).

3. Vaccines containing liver stage antigens

3.1. Cell-traversal protein for ookinetes and sporozoites (CelTOS)

3.1.1. Structure and antigenicity

As its name suggests, CelTOS is expressed in both the ookinetes and sporozoite stages of malaria, and functions in cell transversal [135]. This protein is highly conserved across multiple

species of *Plasmodium* including those that cause malaria in rodents (*P. berghei*) and primates (*P. falciparum* and *P. vivax*) [135]. Identification of CelTOS (182 AA—GenBank: AAN36249.1) as a potential protective antigen built upon a previous proteomic analysis identifying expressed proteins in specific phases of the *P. falciparum* lifecycle [62]. Using prototypical HLA supertypes, algorithms were developed to identify potential peptides that could bind to HLA supertypes, and peptides from 27 proteins were screened for stimulating PBMCs from volunteers vaccinated with irradiated sporozoites and subsequently challenged with infection by *P. falciparum* [55]. From these, CelTOS was identified as stimulating the highest number of effector T cells producing IFN-γ overall [55]. Subsequently, Kaiser et al. identified CelTOS using a suppression-subtractive hybridization approach in *P. yoelii* sporozoites versus merozoites [58].

In malaria-naive volunteers infected with irradiated sporozoites, peptides from CelTOS stimulate IFN-γ production from effector T cells in eight out of 12 volunteers [55]. Six out of 35 Ghanaian adults demonstrated effector T cells to CelTOS [136]. In mice immunized with irradiated sporozoites, low levels of IFN-γ producing CelTOS-specific CD8+ T cells are induced [137].

3.1.2. Preclinical and clinical trials of CelTOS vaccine

Vaccines comprised of CelTOS recombinant protein or expression vectors (i.e., DNA and viral vectors) have demonstrated protective efficacy against malaria in mouse models. CelTOS administered as a recombinant protein with the adjuvants Montanide ISA 720 or glucopyranosyl lipid A stable emulsion (GLA-SE) elicited CelTOS-specific antibodies, CD4+ and CD8+ T cells, and protection (10–76% protection, depending on dose and mouse strain) from infective challenge with *P. berghei* in BALB/c and CD1 mice [65, 138, 139]. This protection was demonstrated using homologous and heterologous challenge, demonstrating the unique level of conservation of CelTOS even across species of the parasite [65, 138]. Note that a challenge was not conducted with GLA-SE formulations. In mice vaccinated with recombinant CelTOS, protective immunity did not correlate with the level of antibody production, and passive immunization did not provide significant protection from infection [138]. In one study, self-adjuvanting bacterial vectors expressing CelTOS were used to vaccinate BALB/c mice and sterile immunity (40–60% protection, depending on dose) was demonstrated even though an antibody response to CelTOS was not detected [140]. In these studies, clearance of parasites infecting the liver plays a major role in protective immunity, which is likely potentiated by CD4+ and CD8+ T cells [138, 141]. Protective immunity in BALB/c mice was dependent on the mixed response of both the CD4+ and CD8+ T cells to CelTOS [138, 140, 141]. Studies using viral vectors encoding CelTOS did not demonstrate protective immunity in either BALB/c or CD1 mice, even though an antibody response and moderate levels of CelTOS-specific CD4+ and CD8+ T cells were induced, and control cohorts immunized with CSP in the same prime/boost protocol did demonstrate sterile immunity [33, 137].

Two Phase I trials with CHMI have been performed with a recombinant CelTOS protein produced in *E. coli* (FMP012) formulated with either GLA-SE or AS01B and tested for safety and efficacy (ClinicalTrials.gov: NCT01540474; ClinicalTrials.gov: NCT02174978). Malaria-

naïve adults (18–50 years of age) were vaccinated three times with FMP012 GLA-SE or four times with FMP012 AS01B, and adverse events were monitored. Antibody titers to FMP012 were monitored. Vaccinated volunteers along with nonvaccinated controls were challenged with *P. falciparum* to determine the efficacy of the immune response as determined by time to parasitemia evaluated by blood smear. Results for neither trial have been published.

3.2. Exported protein-1 (EXP-1)

3.2.1. Structure and antigenicity

Exported protein 1 (EXP-1) (162 AA—GenBank: AAN35808.1) was discovered by screening an *E. coli* expression library of blood stage antigens using a monoclonal antibody to *P. falciparum* and was designated antigen 5.1 (Ag5.1) [142]. It was subsequently rediscovered by other screening approaches and designated circumsporozoite-related antigen (CRA), EXP-1, QF116, and *P. yoelii* hepatocyte erythrocyte protein 17 (HEP17) [143–147]. Adults in malaria-endemic regions produced antibodies to the protein and EXP-1-dependent CD4+ and CD8+ T cells [37, 38, 143]. EXP-1 was incorporated in some of the earliest subunit vaccines for malaria tested in humans [148], and peptides from this protein continue to be incorporated into vaccines for malaria, such as ex23 used in ME-TRAP [44].

EXP-1 is conserved across strains of specific *Plasmodium* species [143, 147]. The demonstration that this protein elicited an immune response that provided protection across a diverse spectrum of murine malaria strains in challenged mice [149] led to analysis identifying supertype-peptides that elicited response from a variety of HLA types [37]. In addition to being a vaccine target, the protein is a glutathione S-transferase that has been associated with artesunate metabolism, making it a potential drug target for malaria [150].

The immune responses of people in malaria endemic regions demonstrate that EXP-1 elicits antibody and IFN-γ responses from CD4+ and CD8+ T cells. Indonesian and African adults had detectable antibodies that react with EXP-1 [151–153]. In African children in malaria endemic regions, the presence of antibody responding to an EXP-1 peptide encompassing amino acids 101–162 were associated with decreased infection [154]. Low level IFN-γ and CD4+ T cells were seen in response to EXP-1 in West Africans, although CD8+ T cells were not detected [151]. A similar limited response of CD8+ T cells and low level CD4+ T cells by Kenyans was noted with only one peptide, EXP-10, stimulating CD8+ T cells [37, 38]. Adults from Gambia and Tanzania with natural immunity to malaria develop EXP-1-dependent CTL and the EXP-1 peptide EX23 has also been shown to elicit a CTL response [155]. In another study, African children naturally exposed to malaria only developed low levels of EXP-1-dependent IFN-γ expression [156]. However, the peptides used for this study (EXP2, EXP80, and EXP91) were previously demonstrated to give no CD8+ T cell response in African adults [37], and may not reflect the potential for EXP-1 as a vaccine component. This potential is demonstrated in Caucasian adults, that when immunized with irradiated sporozoites developed EXP-1-dependent CD8+ T cells as well as CD4+ T cells [37, 55].

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3.2.2. Preclinical and clinical trials of EXP-1

EXP-1 provided protection from malaria in a mouse model where protection was dependent on the development of CD8+ T cells and expression of IFN-γ or nitric oxide [149]. This study resulted in an early emphasis on CD8+-dependent IFN-γ production in the development of subunit vaccines against malaria.

5.1-(NANP)19 is a recombinant protein expressed in *E. coli* that contains EXP-1 fused with 19 repeats of the tetramer NANP from CSP [157]. Thirteen adults were vaccinated subcutaneously with 5.1-(NANP)19 using two or three immunizations containing 50 or 400 μg of protein without adjuvant [148]. The vaccine was safe and only caused reactions at the site of injection. All volunteers developed antibody to the NANP peptide, and six developed 5.1-(NANP)19-spectific effector T cells. Seven volunteers were subsequently challenged with *P. falciparum* by bites from infected mosquitoes. While all seven volunteers developed parasitemia, one did not develop symptoms of malaria.

In a study with 194 semiimmune children (6–12 years old) immunized with 5.1-(NANP)19, all but eight children had considerable levels of antibody to EXP-1 [158]. None of the nonvaccinated children developed malaria in this study over the 12 weeks of observation, and the protective efficacy of the vaccine could not be determined.

EXP-1 has been tested in a number of clinical trials as a component of multiantigen vaccines: EP1300, L3SEPTL, ME-TRAP, and MuStDP5. See the discussion in Section 5, **Table 3**.

3.3. Liver-stage antigen-1 (LSA-1)

3.3.1. Structure and antigenicity

Liver stage antigen 1 (LSA-1) is expressed after parasites have invaded hepatocytes and antigen accumulates in the parasitophorous vacuole [52, 159]. The function of this antigen is not known. LSA-1 was discovered using antisera from volunteers who remained on chloroquine while in a malaria endemic region to obtain antibodies to the liver stage of malaria. The antisera were used to screen recombinant DNA expression libraries [52]. Subsequently, a peptide ls94 from LSA-1 was found to bind to HLA-B53, which is MHC class I associated with resistance to severe malaria in West Africa. This indicated that class-I restricted CD8+ T cells are important for protective immunity against malaria in Africans [160, 161].

LSA-1 (UniProtKB/Swiss-Prot: Q25893) is a 1909 amino acid protein from the *P. falciparum* strain NF54 [162]. Even though strain 3D7 is derived from NF54 [163], the 3D7 strain produces a protein that is 1162 amino acids in length. The amino terminal and carboxyl terminal ends of the protein are nonrepetitive, whereas the middle of the protein contains multiple repeats (**Figure 2**). This repeat region results in significant variation of the protein between strains of *P. falciparum* [162].

LSA-1 antigen stimulates IFN-γ producing T cells, composed of CD4+ and CD8+ T cells, and CTL was demonstrated from volunteers in regions with endemic malaria [36, 37, 151, 164–166]. The immune response to LSA-1 typically increased with age and exposure to the parasite

[165–168]. In Gabonese children, LSA-1-dependent IFN-γ producing T cells were correlated with reduced disease severity [169]. Further, naturally infected individuals often express antibodies to LSA-1, targeting primarily the central repetitive region of the protein [170], and antibody to LSA-1 is associated with a reduction in disease severity [171–173].

Immunization of malaria-naive volunteers with irradiated sporozoites produced an immune response to LSA-1 that included CD4+ and CD8+ T cells, and a weak antibody response [37, 38, 55, 174]. However, when volunteers were challenged with infection, the volunteers who responded to LSA-1 were not protected from malaria [55]. Further, in malaria-naive volunteers immunized by exposure to bites from infected mosquitoes while receiving chloroquine, 74% of the volunteers produced antibodies to LSA-1; however, the humoral response did not provide sterile protection from malaria [175].

3.3.2. Preclinical and clinical trials of LSA-1 vaccine

Rodent strains of *Plasmodium* do not contain orthologs of LSA-1 [64, 176], and animal models evaluating LSA-1 are limited to NHPs. Analysis of immunity to peptides from LSA-1 in combination with LSA-3, SALSA, and STARP in NHPs has been the focus of several studies wherein immunity to the peptides is altered by lipidating the peptides or incorporating the peptides in Montanide ISA-51 [66, 177, 178]. High levels of B cells and CD4+ T cells producing IFN-γ in response to LSA-1 were demonstrated. One of the peptides for LSA-1 (LSA1-J) elicited CD8+ CTLs. The NHPs were not challenged with infection by *Plasmodium*, so the impact of this immune response is not known.

Analysis of LSA-1 vaccination using prime/boost approaches using DNA viral vehicles is limited. A prime boost of LSA-1 in chimpanzee adenovirus 63 (ChAd63) and modified vaccine Ankara (MVA) in BALB/c and CD-1 mice demonstrated sterile protection using a chimeric strain of *P. berghei* expressing the *P. falciparum* LSA-1 protein [33]. Seven out of eight mice, for both the BALB/c and CD-1 mice, demonstrated a significant delay in time to onset of parasitemia. LSA-1-dependent IFN-γ production by CD4+ and CD8+ T cells, and antibodies were detected; however, the mediators for protection were not clear. In BALB/c mice where CD4+ or CD8+ T cells were depleted, protection was not dependent on CD8+ T cells; however, results suggested that CD4+ T cells play a significant role. Analysis of the immune response developed in rhesus monkeys using DNA priming and pox virus boosting (using the nonreplicating canary pox virus ALVAC-Pf7) was carried out using an antigen cocktail containing LSA-1, CSP, TRAP, AMA1, and MSP1 [179]. This resulted in a predominant CD4+ T cell response with secretion of IL-2. Low-level IFN-γ production was attributed to CD4+ T cells and CD8+ T cells were low and only present in animals with high CD4+ T cell responses. No antibodies to LSA-1 were detected. The animals were not challenged with infection by *Plasmodium*, so the impact of this immune response is not known.

Recombinant protein LSA-NRC was developed to assess efficacy of an LSA-1 vaccine in the clinic. LSA-NRC incorporates *P. falciparum* NF54 LSA-1 nonrepetitive N-terminal and C-terminal regions, and includes two 17-amino-acid repeats from the repetitive central region of the protein (**Figure 3**). This antigen was administered with AS01B (a liposomal formulation with MPLA and QS-21) in rhesus monkeys and elicited high titers of antibodies and CD4+ T

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cells producing IL-2 alone or IL-2 with IFN-γ, but CD8+ T cells were not detected [180]. The NHPs were not challenged with infection by *Plasmodium*, so the impact of this immune response is not known.

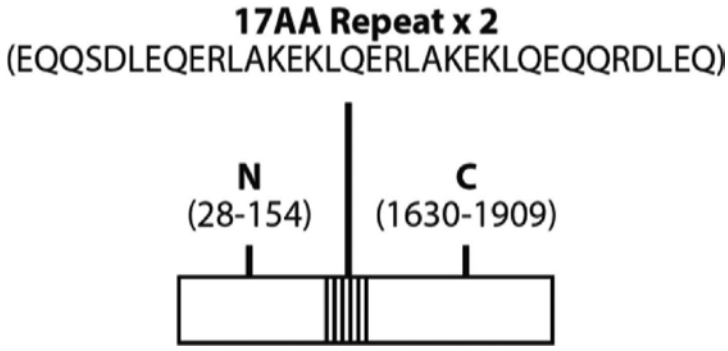

Figure 3. Diagram of LSA-NRC (adapted from [181]).

Phase I trials with CHMI were carried out in malaria naïve volunteers using a recombinant LSA 1 protein (FMP011). LSA-NRC adjuvanted with either AS01B or AS02A was well tolerated, safe, and immunogenic at both low and high doses of the antigen [64]. All volunteers developed antibodies after the first dose of the vaccine. Vaccinated volunteers developed LSA-1 specific CD4+ T cells following the second dose. The low dose of LSA-NRC, with either AS01B or AS02A, resulted in LSA-1-dependent CD4+ T cells that produced both IL-2 and IFN-γ. However, the LSA-1 high-dose groups had fewer CD4+ T cells and little to no IFN-γ response relative to the low-dose cohort. None of the groups produced LSA-1-dependent CD8+ T cells. These results were similar to the immune response seen in the preclinical studies in mice (BALB/c and A/J mice) and rhesus monkeys [180, 182]. Only the high-dose groups were challenged along with nonvaccinated volunteers using the homologous 3D7 strain of P. *falciparum*. All 22 volunteers that were challenged became parasitemic without delayed onset of the erythrocytic infection.

Several clinical trials of multiantigen pre-erythrocytic vaccines that included LSA-1 as an antigen (EP1300, L3SEPTL, ME-TRAP, MuStDP5, and NYVAC-Pf7) have been conducted and are discussed in Section 4, **Table 3**.

3.4. Liver-stage antigen-3 (LSA-3)

3.4.1. Structure and antigenicity of LSA-3

While LSA-3 is included with the liver-stage antigens, some report it is actually a blood stage antigen [183]. Using malaria-naive volunteers immunized with irradiated *P. falciparum*, LSA-3 was identified by comparing antibody response of protected and nonprotected volunteers to a recombinant DNA expression library [53].

LSA-3 is composed of three nonrepeating regions (NR-A, NR-B, and NR-C) flanking two short repeat regions (R1 and R3) and one long repeat region (R2) (see **Figure 4**) [53]. The nonrepeat regions are well conserved across geographically diverse strains of *P. falciparum* [184]. The most significant variation is in the repeating regions, but this is due to the organization and the number of repeating subunits rather than composition of the repeating regions [184]. Vaccine development draws upon the protein from *P. falciparum* strains T9/96 and 3D7 (1586 AA GenBank: ACT22567.1).

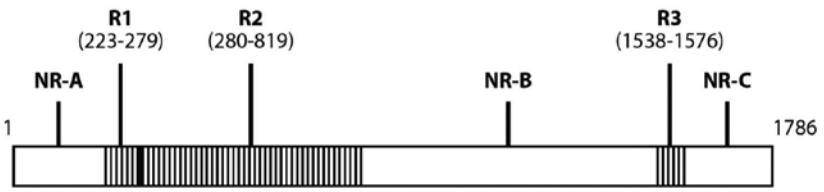

Figure 4. Diagram of the domains of LSA-3 (adapted from [185]).

In individuals exposed to *P. falciparum*, LSA-3 elicits antibodies, and CD4+ and CD8+ T cells. West African adults demonstrated CD4+, and class I–LSA-3-dependent CTLs [151, 155]. Antibodies to LSA-3 were demonstrated in children from West Africa, and increased with age [171, 185], and malaria-naive Europeans develop antibodies to LSA-3 when immunized with irradiated sporozoites [53].

3.4.2. Preclinical and clinical trials of LSA-3

Rodent strains of *Plasmodium* do not contain orthologs of LSA-3 [176], and animal models evaluating LSA-3 are limited to NHPs. A murine model with the infectious challenge using a strain of *P. berghei* that was genetically modified to contain the *P. falciparum* LSA-3 gene has been reported [33]. BALB/c and CD1 mice were primed with ChAd63 and boosted with MVA encoding LSA-3, but sterile immunity was demonstrated in only 13% of the BALB/c mice. The BALB/c mice developed antibodies and moderate levels of PBMCs producing LSA-3-specific IFN-γ were developed. Both CD4+ and CD8+ T cells were produced against the antigen, and CD8+ T cells predominated.

Vaccination with LSA-3 has been shown to elicit sterile immunity in chimpanzees [53]. This study demonstrated sterile immunity using various forms of LSA-3, albeit only one chimpanzee was used for each specific antigen. LSA-3 was expressed as GST-fused recombinant peptides in three segments: DG (encompassing AA 1–60), NN (encompassing AA 369–447), and PC (encompassing AA 869–1786). The mixture of these three peptides (designated as LSA-3 GST) elicited sterile immunity when administered with the adjuvant SBAS2 (SBAS2 is an adjuvant containing MPLA and QS21) or with Montanide ISA 51. The synthetic lapidated peptides CT1, NR1, NR2, and RE were also evaluated. Vaccination with NR2 alone elicited sterile immunity. Further, NR2 administered with NR1 and Montanide ISA 5, or with NR1 and CT1 with Montanide ISA 51 also elicited sterile immunity. Studies in chimpanzees using the

same LSA-3 peptides in combination with peptides derived from LSA-1, STARP, and SALSA demonstrated that the LSA-3 peptides elicited IFN-γ production with a mixed response of CD4+ and CD8+ T cells [66].

Later studies in chimpanzees immunized with plasmid expressing the nearly full length LSA-3 also demonstrated LSA-3 provided protection, with three of the four immunized chimpanzees demonstrating sterile immunity [186]. In this study, only low levels of IFN-γ were detected from a mixed class I and class II response, and no antibodies were detected. Studies in *Aotus* monkeys immunized with recombinant LSA-3 without adjuvant also developed low but sustained levels of antibodies to LSA-3, and LSA-3 dependent IFN-γ production; however, neither correlated with protection [187]. Subsequent challenge with *P. falciparum* demonstrated that three of the five immunized monkeys were sterilely protected. Interestingly, immunofluorescence assay titers to *P. falciparum* sporozoites were highest in monkeys that were protected. These results were repeated when *Aotus* monkeys were immunized using two peptides of LSA-3, amino acids 100–222 in the amino-terminal nonrepeat region, and amino acids 501–596 in the repeat-2 region of the protein from *P. falciparum* strain T9/96 [187]. In this later study, monkeys were immunized with the recombinant peptides adjuvanted with AS02, eliciting antibody and high IFN-γ responses with sterile immunity in four out of four immunized animals.

A Phase I clinical trial with challenge evaluating the safety and efficacy of recombinant LSA-3 protein adjuvanted with aluminum hydroxide or Montanide ISA 720 was carried out, but the results of the study have not been published [188].

Clinical trials for multistage pre-erythrocytic vaccines that contain LSA-3 include L3SEPTL, ME-TRAP, and MuStDP5 and are discussed in Section 4, **Table 3**.

3.5. Sporozoite threonine-asparagine-rich protein (STARP)

3.5.1. Structure and antigenicity

STARP (GenBank: CAA81224.1) was identified by screening a lambda library with antibodies developed in malaria exposed missionaries under chloroquine treatment [51]. It is a 604-amino acid protein with a central hydrophilic region (amino acids 85–489) that contains a complex repetitive structure that can vary in size between different strains of *P. falciparum*. Two peptides from this repetitive region (Rp10 and Rp45) have been evaluated in vaccines [51]. Even with variation in the repetitive structure, the protein is highly conserved across a variety of geographically separated strains [51, 189].

People in malaria endemic regions produce antibody to STARP [190–192], with the percentage of the population related to the rate of exposure to malaria [193]. The main STARP antibody response in Africans naturally exposed to malaria or volunteers immunized with irradiated sporozoites was to the Rp10 peptide, and purified antibody prevented 90% of the sporozoites from infecting human liver cells *in vitro* [193]. In West African children, the presence of antibody to STARP was associated with protection from malaria [171]. A conserved HLA class I epitope eliciting a CTL response in adults naturally infected with *P. falciparum* has been

identified and was incorporated in the multiple epitope used as part of the vaccine ME-TRAP [36, 41] (Section 4, **Table 3**).

3.5.2. Preclinical and clinical trials of STARP

A limited number of T cell epitopes have been identified in STARP. The conserved epitope st8 was demonstrated to elicit a CD8+ T cell CTL response in an African adult exposed to *P. falciparum* [36]. The Rp10 epitope is both a B cell and class I T cell epitope [189]. However, in a NHP model, the Rp10 epitope elicits low levels IFN-γ response but no detectable CTL; note that the NHPs were not challenged with infection in these studies [66, 178].

Homologues of STARP have been identified in *P. yoelii* (PY00217 and PY05105) and *P. reichenowi* (PRCDC_0700500) [176, 189]. However, no studies of animals vaccinated with STARP and challenged with infection are published.

Clinical trials for multiantigen vaccines that include STARP, L3SEPTL, and ME-TRAP, are discussed in Section 4, **Table 3**.

3.6. Thrombospondin-related anonymous protein (TRAP)

3.6.1. Structure and antigenicity of TRAP

The TRAP protein was initially identified based on peptide motifs in common with thrombospondin and properdin [194]. Subsequently, it was demonstrated that TRAP contributed to the binding of sporozoites to hepatic cells [195], and antibodies to the protein block binding of sporozoites to hepatic cells [196]. In addition to TRAP referring to "thrombospondin-related anonymous protein," the literature refers to TRAP as "thrombospondin-related adhesion protein" [42] and as sporozoite surface protein-2 (SSP2) [196]. TRAP antigen from *P. falciparum* T9/96 (559 AA-PRF: 226137) differs by 6% from the amino acid sequence of TRAP in the 3D7 strain (574 AA-CAD52497.1). The major difference between the strains is the protein from the 3D7 strain contains hepta-repeat of the PPN sequence while T9/96 only contains a single PPN.

Early development of TRAP as a component in a malaria vaccine focused on identifying peptides that were recognized by MHC 1 and elicited a CTL response from individuals exposed to the parasite [36–38, 41, 197, 198]. Evaluation of the immune response to TRAP in naturally infected African donors demonstrated CD4+ and CD8+ T cells. Gambian and Kenyan adults demonstrated CD8+ T cell-dependent CTL activity against TRAP [36–38, 151, 197]. In a study in Kenya, TRAP-dependent IFN-γ producing T cells correlated with reduced disease severity in children [199]. Additionally, it has been demonstrated that a CD4+ T cell response dominated over a CD8+ T cell response to TRAP in Kenyan children and adults, and the CD4+ T cells reactive with TRAP were correlated with reduced risk of clinical malaria [200].

Analysis of the immune response to TRAP in malaria-naive volunteers immunized with irradiated sporozoites (using the 3D7 clone of *P. falciparum* strain NF54) demonstrated CD8+ T cell-dependent CTL activity against TRAP [37, 198], and TRAP-specific CD4+ T cells [38]. In a separate study using 12 malaria-naive Caucasian male volunteers immunized with irradiated

sporozoites, TRAP was recognized by five of the volunteers and resulted in the development of TRAP responsive CD4+ T cells, CD8+ T cells, and antibodies [55].

While antibodies are recognized as important in protection from the erythrocytic phase of malaria and reducing the severity of disease, their role in providing sterile protection in the liver-stage is controversial. *In vitro*, antibodies to TRAP can block binding of sporozoites to human hepatocytes, and it has been postulated that antibodies could help to prevent infection of the liver [201]. In a study using malaria-naive volunteers infected with *P. falciparum* while receiving chloroquine to provide sterile immunity, volunteers did not produce antibodies to TRAP or detectable memory B cells [175]. A recent study, using malaria-naive volunteers vaccinated with irradiated sporozoites and subsequently challenged with the homologous strain of *P. falciparum* (3D7 clone of NF54) correlated the magnitude of the antibody response to TRAP with sterile protective immunity [54]. In this study, both sterilely protected individuals (six volunteers) and individuals who developed blood-stage parasitemia (five volunteers) developed antibodies to TRAP and other *P. falciparum* antigens, but the magnitude of the immune response was significantly higher in the protected volunteers. In a similar but separate study using 12 volunteers immunized with irradiated sporozoites, while a subset of the volunteers responded to TRAP, the immune response to TRAP was associated with volunteers who did not develop sterile immunity [55].

3.6.2. Preclinical and clinical trials of TRAP vaccine

Recent studies have tested TRAP alone and in combination with RTS,S in malaria naive adults [202]. The expressed protein was a truncated form of TRAP consisting of amino acids 26–511 with an additional hepta-histidine at the carboxyl-terminus. Volunteers immunized with TRAP in AS02 developed antibodies and modest CD4+ and CD8+ T cell responses (46 SFU/10^6 PBMCs). In an effort to determine if there was a synergistic effect of immune responses to TRAP and CSP, a clinical study with CHMI was conducted where volunteers were immunized with two 25 μg doses of TRAP or two doses of both TRAP and RTS,S (25 and 50 μg, respectively) in AS02. Following challenge, 1/11 volunteers (9%) developed sterile immunity in the cohort vaccinated with TRAP and RTS,S with no significant delayed patency in remaining volunteers. The level of protective efficacy was lower than was previously reported for immunization with RTS,S alone, suggesting potential antigenic competition.

Clinical trials of multiantigen vaccines containing TRAP, including EP1300, L3SEPTL, ME-TRAP, MuStDP5, and NYVAC-Pf7 are discussed in Section 4, **Table 3**.

4. Multiantigen pre-erythrocytic vaccines

Early studies with multiantigen vaccines were based on the rational that a combination of antigens would elicit a broad immune response more comparable to that elicited by whole sporozoite vaccine. A number of multiantigen vaccines comprised of DNA, polyprotein, and recombinant viral vectors containing combinations of epitopes or entire pre-erythrocytic antigens have been tested in clinical trials (listed in alphabetical order).

4.1. EP1300

EP1300 is a multivalent DNA vaccine composed of a total of 38 CTL and 16 HTL epitopes derived from TRAP, CSP, EXP-1, and LSA-1 expressed as a single protein, and the NANP repeating epitope from CSP for antibody development (**Figure 5**). The epitopes are linked using spacers that facilitate proteolytic processing of the protein into individual epitopes in the body; while this worked in animals, processing may not have occurred correctly in humans.

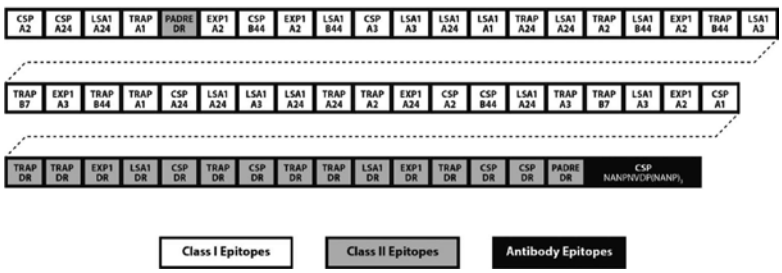

Figure 5. Arrangement of epitopes from CSP, EXP-1, LSA-1, and TRAP in EP1300.

A Phase Ia dose-escalating study in healthy malaria-naive adults (18–40 years of age) was initiated in 2010 to evaluate the safety and immunogenicity of EP1300. EP1300 was delivered using electroporation. This trial was completed in 2015 [203]. The trial demonstrated the vaccine was safe and well tolerated but did not elicit a significant immune response in the volunteers (Unpublished Results, ClinicalTrials.gov: NCT01169077).

4.2. L3SEPTL polyprotein vaccine

L3SEPTL polyprotein is comprised of six pre-erythrocytic-stage antigens, LSA-3, STARP, EXP-1, Pfs16, TRAP, and LSA1-N/C, linked together to produce a 3240-aa-long polyprotein (**Figure 6**) [204]. In this construct, LSA-1 is modified so that only one repeat is included flanked by the conserved amino-end (amino acids 1–148) and the carboxyl-end (amino acids 1523–1909). The nucleic acid sequence expressing the recombinant fused protein is delivered as plasmid DNA using the human cytomegalovirus promoter, or in the viral vehicles MVA and Fowlpox (FP9), to provide the vaccines PP, MVA-PP, and FP9-PP, respectively.

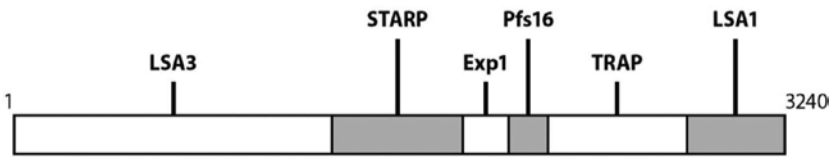

Figure 6. Diagram of the L3SEPTL fusion protein (adapted from [204]).

The MVA-PP and FP9-PP vaccines were evaluated in a Phase I with challenge dose-escalation human clinical trial using malaria-naive volunteers [205]. Generally, the vaccine was well tolerated, but the immunity was lower than expected. Priming with MVA resulted in a stronger immune response than did FP9, and when FP9 was used for priming (FP9/FP9/MVA), the immune response was not significantly elicited until the MVA vehicle was used to deliver the antigens [205]. This was in contrast with the results seen in the preclinical study using FP9-PP for the priming. When delivered using MVA priming, all six proteins elicited IFN-γ-producing T cells, with stronger and more frequent responses seen for LSA-3, LSA-1, TRAP, and STARP. However, the number of IFN-γ-producing T cells was lower than had been reported in other studies [205]. Following challenge, none of the 15 vaccinated volunteers demonstrated protection or delayed onset of disease relative to the six nonvaccinated volunteers.

4.3. ME-TRAP

The most extensively tested multiantigen vaccine, ME (multiepitope)-TRAP vaccine, was designed to elicit T cell responses to target the hepatic stages of the malaria parasite. Nucleic acid encoding an ME string was designed to include CD8+ and CD4+ T cell epitopes from TRAP, CSP, EXP-1, LSA-1, LSA-3, and STARP (**Table 2**) [36, 41, 197, 198]. The ME were fused in-frame to the nucleic acid sequence encoding the entire TRAP antigen from *P. falciparum* strain T9/96, encoding a polypeptide of 789 amino acids referred to as multi-epitope thrombospondin-related adhesion protein (ME-TRAP) [44].

Antigen	Designation	Amino acid sequence	Epitope
CSP	cp6	MNPNDPNRNV	HLA class I antigens
CSP	cp26	KPKDELDY	
CSP	cp39	YLNKIQNSL	
EXP-1	ex23	ATSVLAGL	
LSA1	ls6	KPIVQYDNF	
LSA1	ls8	KPNDKSLY	
LSA1	ls50	ISKYEDEI	
LSA1	ls53	KSLYDEHI	
LSA3	la72	MEKLKELEK	
STARP	st8	MINAYLDKL	
TRAP	tr26	HLGNVKYLV	
TRAP	tr29	LLMDCSGSI	
TRAP	tr39	GIAGGLALL	
TRAP	tr42/43	ASKNKEKALII	
CSP (*P. berghei*)	pb9	SYIPSAEKI	Mouse MHC class I antigen
CSP	NANP	NANPNANPNANPNANP	B cell epitopes
TRAP	TRAP-AM	DEWSPCSVTCGKGTRSRKRE	Heparin binding motif
CSP	CSP	DPNANPNVDPNANPNV	Class II antigens (BCG and Tetanus toxin are not malarial antigens)
BCG		QVHFQPLPPAVVKL	
Tetanus toxin	QFIKANSKFIGITE		

Table 2. Peptides used in the multi-epitope domain of ME-TRAP [41].

Vaccine	Antigens (adjuvants)	Delivery regimen	Efficacy (# protected/# participants)	Immune response	Population	References
L3SEPTL (peptide)	LSA, STARP, Exp1, Pfs16, TRAP, and LSA1	FFM	0% protection (0/8)	85 SFU/10^6 PBMCs	Malaria naïve adults	[205]
		MMF	0% protection (0/7)	96 SFU/10^6 PBMCs		
ME TRAP	TRAP and peptides from CSP, LSA-1, LSA-3, EXP-1, STARP expressed as a single protein.	DDDMM	0% protection (8/8 delayed patency)	158-316 SFU/10^6 PBMCs	Malaria Naïve adults	[44]
		DDMM	0% protection, (6/6 delayed patency)	234 SFU/10^6 PBMCs		
		DDD	0% protection (0/5)	33 SFU/10^6 PBMCs		
		MMM	0% protection (0/4)	44 SFU/10^6 PBMCs		
		DDM	12.5% protection (1/8 sterile immunity, 7/8 delayed patency)	423 SFU/10^6 PBMCs; low titer antibodies	Malaria naïve adults	[42]
		DDM	10% protective efficacy relative to control group	250 SFU/10^6 PBMCs	Gambian adults	[219]
		FFM	12.5% protection (2/16 sterile immunity, 14/16 delayed patency)	430 SFU/10^6 PBMCs, mixture of CD4+ and CD8+ T cells	Malaria naïve adults	[45, 207]
		MMM	0% protection (0/4)	0 SFU/10^6 PBMCs		
		FM	0% protection (0/5)	380 SFU/10^6 PBMCs, mixture of CD4+ and CD8+ T cells		
		MF	0% protection (0/5)	100 SFU/10^6 PBMCs, mixture of CD4+ and CD8+ T cells		
		DDMF	0% protection (4/4 delayed patency)	200 SFU/10^6 PBMCs, CD4+ T cells		
		DDFM	0% protection (0/3)	300 SFU/10^6 PBMCs, CD4+ T cells		
		FFM	0% protection (73% infection in vaccines, 80% infection in controls; no statistically significant decrease)	107 SFU/10^6 PBMCs	Kenyan children	[210]
		CM	21% protection (3/14 sterile immunity, 5/14 delayed patency)	1300 SFU/10^6 PBMCs, CD4+ and CD8+ T cells producing IFN-γ, IL2, TNF-α(protection associated with CD8+ IFN-γ producing T cells)	Malaria naïve adults	[212]
		CM	13% protection (2/15 sterile immunity, 5/15 delayed patency)	2000 SFU/10^6 PBMCs, and antibodies detected	Malaria naïve adults	[213]

Vaccine	Antigens (adjuvants)	Delivery regimen	Efficacy (# protected/# participants)	Immune response	Population	References
		CM	67% protection (18% infection in vaccines, 47% infection in controls)	1450 SFU/10^6 PBMCs, CD4+ and CD8+ T cells producing IFN-γ, IL2, TNF-α	African adults	[105, 214]
MuStDO5	TRAP, CSP, EXP1, LSA1, and LSA3	DDD	0% protection (0/8)	For both regimens: class I—41 SFU/10^6 PBMCs; class II—59 SFU/10^6 PBMCs, no antibodies detected	Malaria naïve adults	[215, 216]
		DDD & plasmid with hGM-CSF	0% protection (0/23)			
NYVAC-Pf7	CSP, TRAP, LSA-1, MSP1, SERA, AMA1 and Pfs25	NNN	3% protection (1/35 sterile immunity, 34/35 delayed patency)	9/38 volunteers developed CTL to TRAP; 2/38 volunteers developed CTL to LSA1; 50% of volunteers developed antibody to TRAP and LSA1	Malaria naïve adults	[218]

*Regimens are the sequence of immunization using the vehicles D (plasmid DNA), F (attenuated fowlpox virus FP9), M (modified vaccinia virus Ankara), C (chimpanzee adenovirus 63), and N (attenuated vaccinia virus NYVAC) to provide gene sequences encoding the described antigens.
**T cells isolated by FAC, antigen-dependent T cell count not provided. SFU refers to antigen-specific IFN-γ producing T cells.

Table 3. Summary of the efficacy of multi-antigen pre-erythrocytic vaccines tested in clinical trials.

Expressing the ME-TRAP antigen *in situ* using DNA vaccines (plasmids) or viral vehicles has been employed to provide low-cost vaccination. While results in animal models using plasmids to deliver antigens were promising, the immunity elicited in humans was typically low. Heterologous prime/boost combinations, wherein the antigen remained constant but was delivered using different vehicles, reduced the development of immunity to the vehicle while provided a boost to the immune system with the target antigens [206]. In humans, the order of vehicle used for immunization was demonstrated to affect overall immune response [45, 207]. Thus, studies have focused on identifying the appropriate combination and order of vehicles that can be used to produce an elevated and expanded immune response. Plasmids encoding ME-TRAP only elicited a low-level immune response [44], and subsequent efforts have focused on viral vehicles for both priming and boosting (**Table 3**).

The MVA strain has proven to be a strong vehicle for boosting, and clinical trials using ME-TRAP subsequently focused on identifying an optimal priming vehicle and dosing regimen (see **Table 3**). Using an attenuated fowlpox virus vehicle (FP9) with MVA elicited strong immunity in malaria-naive volunteers and limited protection [207], but provided only low-level immunity in people in malaria-endemic regions without providing significant protection [208–210] (see **Table 3**). The recent addition of a chimpanzee adenovirus 63 (ChAd63) for priming has provided encouraging results (see **Table 3**). AdCh63 priming elicits both CD4+ and CD8+ T cells at much higher levels than DNA or FP9 priming [211], and also results in a

much higher proportion of IFN-γ-secreting monofunctional CD8+ T cells that were correlated to sterile protection [212]. This combination has provided protection from infection in malaria-naive volunteers comparable to the immunity provided by CSP using the same delivery platform [212, 213]. Further, ME-TRAP using the ChAd63/MVA regimen elicited a strong immune response and provided significant protection in Africans who had prior exposure to malaria [43, 214]. Using a Cox regression analysis, vaccine efficacy was 67% during the 56 days of monitoring.

4.4. Multistage DNA vaccine operation five antigens (MuStDO5)

The MuStDO5 cocktail is composed of sequences encoding the antigens TRAP, CSP, EXP-1, LSA-1, and LSA-3 using sequences based on *P. falciparum* strain 3D7 [215, 216]. LSA-1 only encodes the nonrepetitive amino and carboxyl ends of the protein (462 amino acids), with the repetitive 727 amino acid middle domain removed. Each antigen is encoded on a separate plasmid using the same vector VCL-25 (Vical Inc., San Diego, CA, USA), with expression controlled by the promoter/enhancer of the human cytomegalovirus. The cocktail of plasmids is used for vaccination to provide each of the five antigens. In this study, the impact of including a plasmid expressing human granulocyte-macrophage colony-stimulating factor (hGM-CSF) as an immune enhancer was evaluated. This vaccine is safe and well tolerated. However, protective immunity was not demonstrated, and the addition of hGM-CSF decreased the response to class I epitopes.

4.5. NYVAC-Pf7 attenuated vaccinia virus

NYVAC-Pf7 is composed of an attenuated vaccinia virus NYVAC engineered to express seven antigens (Pf7) to target pre-erythrocytic, blood stages, and transmission stages of the parasite [217]. The seven antigens include three pre-erythrocytic antigens (CSP, TRAP, and LSA-1), three blood stage antigens (MSP1, SERA, and AMA1), and the Pfs25 antigen found on the sexual stage ookinete. CSP, TRAP, LSA-1, AMA1, and Pfs25 are derived from *P. falciparum* 3D7 strain. The *lsa-1* gene was modified to produce a protein linking amino acids 1–458 with 1630–1909, removing the sequence encoding the repeat region of the protein. Each gene is under a separate poxvirus promoter and expressed as a separate protein as follows: CSP under H6, TRAP under 42K, LSA-1 under C10LW, MSP1 under H6, SERA under 42K, AMA1 under I3L, and Pfs25 under I3L (**Figure 7**). Each gene was inserted into defined sites in the genome of the attenuated vaccinia strain NYVAC by *in vivo* recombination to produce NYVAC-Pf7.

A Phase I/II clinical trial was carried out with malaria-naive adults [218]. Volunteers were immunized with either high dose (1×10^8 pfu) or low dose (1×10^7pfu) of NYVAC-Pf7 at 0, 4, and 26 weeks. Vaccination with NYVAC-Pf7 resulted in delayed onset of parasitemia following challenge in all volunteers who received the vaccine, as compared to nonvaccinated volunteers. The CTL response or antibody titer did not demonstrate a relationship to the time of delay to onset of parasitemia. One vaccinated volunteer from the low-dosage group demonstrated sterile immunity. About 50% of the volunteers in each of the dosage groups developed antibodies to TRAP and LSA-1, regardless of the dosage. Low levels of CTL were developed to TRAP, with no statistical significance due to the dosage. A CTL response to LSA-1 was only

seen in the low-dosage group. However, the CTL response did not correlate with the time to onset of parasitemia, and the one volunteer exhibiting sterile immunity did not develop a detected CTL response.

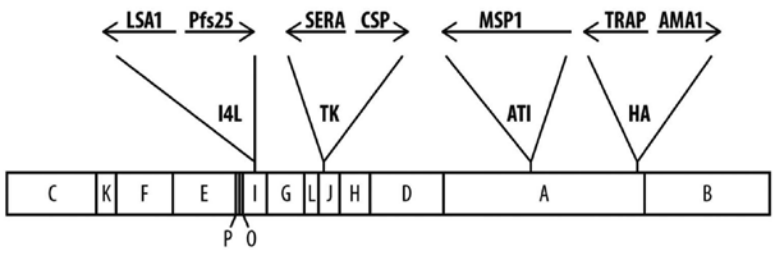

Figure 7. Genomic Organization of the NYVAC-Pf7 (adapted from [217]). The poxvirus promoters are represented with solid boxes.

5. New proteins for pre-erythrocytic malaria vaccines

New malarial proteins have been discovered through screens for pre-erythrocytic phase sporozoites, including identifying mRNA or proteins expressed in specific stages of the lifecycle of the parasite (e.g., sporozoites for the mosquito salivary glands), and typically included monitoring the immune response to the specific proteins using antibodies or T cells from volunteers vaccinated with sporozoites that were either irradiated or suppressed with chemotherapy, as discussed in the Introduction. Section 5 summarizes the preclinical studies carried out focusing on antigens analyzed beyond the initial identifying screen.

5.1. DNA vaccine encoding SAP1

Sporozoite asparagine-rich protein 1 (SAPI) was identified using suppressive-subtractive hybridization to identify *P. berghei* genes that are up-regulated in the sporozoite as it transitions from the mosquito midgut into the salivary gland [60]. In targeted deletion studies, *P. yoelii* SAP1 knockout sporozoites can invade hepatocytes but arrest in early liver stage development [220]. The *P. yoelii* genome sequence encoding the SAP1 protein domain corresponding to amino acids 3063–3227, and flanked by two CpG motifs to enhance the immune response, was cloned into pcDNA3.1(+) [221]. SAP1encoded in plasmid DNA was evaluated for protective immunity against challenge with a homologous strain of *P. yoelii*. BALB/c mice were immunized intramuscularly with 100 μg plasmid three times with 3 weeks between each vaccination. Controls included mice vaccinated with phosphate buffered saline. For the protection study, each group was composed of ten mice. BALB/c mice were challenged by bites from mosquitoes infected with *P. yoelii* 2 weeks after the final immunization. Onset of parasitemia was evaluated using blood smears. Mice without parasitemia 14 days after challenge were considered completely protected. The mice immunized with DNA encoding SAP1 produced SAP1-

specific antibodies and moderate levels of T cells secreting IFN-γ. All 10 of the control mice vaccinated with phosphate buffered saline developed parasitemia. Two of 10 (20%) of the mice vaccinated with pcDNA3.1(+)/SAP1 did not develop parasitemia.

5.2. Adenovirus vectored pre-erythrocytic antigens

This was an exploratory study to evaluate the protective immunity of newly identified pre-erythrocytic-stage antigens, selected from sporozoite and liver stage libraries based on the predicted signal sequences [137]. The *P. yoelii* orthologues of P36p (PY01340; Py52), Ag5 (PY00410; hypothetical protein), Sporozoite Invasion-Associated Protein-1 (PY00455; SIAP1), Kruepple-like protein (PY00839; KLP), and TRAP-like protein (PY01499; TLP), CelTOS or CSP were delivered in 10^{10} virus particles of adenovirus encoding each individual antigen [137]. Protection was evaluated as the parasite burden in the liver following infection. Immunization of female BALB/c (H2-K^d) mice with adenovirus vector (Ad) expressing P36p, CelTOS, or Ag5 elicited a high IFN-γ T cell response that was comparable to the response to CSP. Following challenge, only mice immunized with Ad-CSP reduced the parasite burden. None of the other antigens reduced the burden of parasites as compared to nonvaccinated mice.

5.3. Heterologous prime boost with DNA and vaccinia viral vector

The protective immunity of the individual antigens CSP, Falstatin, PY03661, and UIS3, as well as combinations of the antigens, were evaluated using the *P. yoelii* model [222]. CD1 mice (14 per group) were vaccinated intramuscularly with a priming dose of the DNA vaccine, and boosted with the vaccinia vector vaccine. Parasitemia was evaluated by blood smears following challenge with 300 *P. yoelii* sporozoites. The combination of Falstatin and UIS3 and PY03661 provided protective immunity (57%) against challenge. Heterologous prime boost with CSP protected 36% of the mice. The addition of plasmid expressing murine GM-CSF to immunization scheme did not enhance protective immunity.

5.4. Heterologous prime boost with viral vectors ChAd63 and MVA

Humoral responses in mice were evaluated using recombinant ChAd63 and MVA viral vectors expressing eight individual *P. falciparum* 3D7 antigens [33]. Responses elicited following immunization with five newly identified antigens, upregulated in sporozoite 3 (UIS3), Falstatin, liver-stage associated protein 1 (LSAP1), liver-stage associated protein 2 (LSAP2), and the early transcribed membrane protein 5 (ETRAMP5) were compared to three previously characterized antigens, LSA-1, LSA-3, and CelTOS.

Protective efficacy was evaluated in both BALB/c (inbred) and CD1 (outbred) mice, with each antigen evaluated in a separate cohort of mice using heterologous prime boosts with ChAd63/MVA. Mice were challenged with *P. berghei* transgenic parasites that were modified to express the cognate *P. falciparum* antigen. The immunity and protective efficacy against infection was evaluated for each antigen using nonvaccinated controls for comparison.

The BALB/c mice developed antibodies to all the antigens except LSAP1. High levels of IFN-γ (>1000 SFU/10^6 PBMCs) developed in mice immunized with UIS3 and LSA1. Moderate levels

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of IFN-γ (>250 SFU/10^6 PBMCs) were developed against ETRAMP5, CelTOS, LSAP2, Falstatin, and LSA-3. LSAP1 elicited very low levels of IFN-γ (<100 SFU/10^6 PBMCs). CD8+ T cells predominated for UIS3, LSA-1, and LSA-3 cellular responses, while CD4+ T cells predominated for Falstatin. Highest levels of protection were found in BALB/c or CD1 mice immunized with LSAP2 (85 and 70%, respectively) or LSA-1 (87.5% for both murine strains). Mice immunized with CSP gave 37.5 and 33.3% sterile immunity in BALB/c and CD1 mice, respectively. Strain variation noted, with 30% of CD1, but none of the BALB/c, mice were protected following immunization with TRAP or LSAP1. Minimal or no protection was obtained in mice immunized with ETRAMPS, CelTOS, UIS3, Falstatin, or LSA-3.

6. Conclusions

Trends, advances, and roadblocks for malaria pre-erythrocytic vaccine development identified by the preclinical and clinical studies summarized in this chapter include:

- The hallmarks for protective immunity are not clear and the elements associated with sterile immunity vary in humans. Antibodies, IFN-γ, CD4+ T cells, CD8+ T cells, and cytolytic lymphocytes are believed to play essential roles in immunity, but are not always collectively demonstrated in immunity that protects from infection by *Plasmodium*.
- To achieve effective immunity that induces T cells across a wide population of people will require multiple and diverse T cell epitopes. Many of the studies that identified T cell epitopes in humans demonstrated that, within the limited number of volunteers in a Phase I clinical trial, only a limited subset of individuals respond to any single protein.
- Vaccines that string T cell epitopes from different proteins together in a composite recombinant protein (e.g., multiepitope strings) do not elicit significant T cell responses in humans. This is found even when other proteins in the vaccine do elicit strong immune response.
- For protective immunity in humans, the means and route of administration effect efficacy of the antigens. For example, irradiated sporozoites are more effective by intravenous verses intradermal administration, and TRAP delivered using heterologous vehicles of chimpanzee adenovirus followed by a modified vaccinia virus (heterologous prime boost regimen) provides more effective immunity than immunization using only one of these vehicles, or using other combinations of vehicles.
- Adjuvant formulations remain a critical factor for pre-erythrocytic vaccines, as highly purified subunit vaccine lack the pathogen-associated molecular patterns that trigger the innate immune responses required for initiation of adaptive immunity. Alum has been found to be a poor adjuvant for malaria subunit vaccines in Phase I/II trials. Oil-based adjuvants, e.g., ISA 50 or ISA 720, while enhancing immune responses, have been limited by increased reactogenicity. Precisely targeted adjuvants, such as TLR agonists, have been shown to enhance immunogenicity and protective efficacy of pre-erythrocytic vaccines, and hold promise as future adjuvant formulations.

- While cost competitiveness is a goal for malaria vaccines, it is unlikely that this has been a primary consideration in the early development plan for many candidates as much of the focus is on efficacy. Multiple platforms and approaches have been described in this chapter including DNA-based platforms, viral vehicles, recombinant proteins, and chimeric proteins composed of peptides. In consideration of vaccine design and development, production may be an initial focus to contain costs per dose. This includes consideration for adjuvants and processes to stabilize the vaccine for delivery to the clinic. However, it is not always obvious in early development which approaches may be the most cost effective for commercial vaccine production as efficiencies may be realized through optimizing processes and scaling of production so as to reduce costs significantly relative to the costs to produce research material. Further contributors to the direct cost for vaccination are transportation and storage requirements for the vaccine, the dosage, and number of boosters required to achieve protective immunity. This cost analysis may also need to consider compliance if complex or protracted boosting schedules are required. While development may strive to contain these direct costs, the vaccine still must be effective at preventing the morbidity and mortality caused by malaria.

Acknowledgements

This publication was made possible through support provided by the Office of Health, Infectious Diseases, and Nutrition, Bureau for Global Health, U.S. Agency for International Development, under the terms of the Malaria Vaccine Development Program (MVDP) Contract AID-OAA-C-15-00071, for which Leidos, Inc. is the prime contractor. The opinions expressed herein are those of the author(s) and do not necessarily reflect the views of the U.S. Agency for International Development.

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Chapter 17

Enabling Vaccine Delivery Platforms and Adjuvants for Malaria

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/104405

Abstract

Enabling vaccine delivery platforms and adjuvants with promising attributes for malaria vaccine development are reviewed within the framework of accessibility, efficacy, clinical status, cost, and cold-chain considerations. An emphasis is placed on commercially available platforms and adjuvants including virus-like particle, nanoparticle, microneedle, and mRNA vaccine delivery platforms as well as lipid vesicle, microparticle, and emulsion-based adjuvants. Strategies for addressing complications of vaccine delivery in endemic regions due to concatenate vaccination and infection, and parasite immune avoidance mechanisms are presented. Additionally, recent findings regarding how malaria infection triggers inflammatory pathways and T cell exhaustion along with negative impacts to the development of effective memory responses are described in a context relevant to vaccine development.

Keywords: *Plasmodium falciparum*, malaria, adjuvant, vaccine, delivery platform, VLP, virus-like particle, microneedle, mRNA, nanoparticle, microparticle, liposome, emulsion, nanoemulsion, TLR agonist, T cell exhaustion, PD-1, immune response, CSP

1. Introduction

Efforts to develop an effective malaria vaccine have been ongoing for decades. In order to promote better coordination and acceleration of malaria vaccine development, the World Health Organization (WHO) initiated a process that resulted in the Malaria Vaccine Technology Roadmap, which was first published in 2006 [1] and updated in 2013 [2]. The vision in the roadmap includes development of vaccines for *Plasmodium falciparum* and *P. vivax* to prevent

disease, death, and malaria transmission as part of enabling malaria eradication efforts. As per the updated Malaria Vaccine Technology Roadmap, the goal of a successful vaccine candidate is ≥75% protective efficacy over 2 years with no more than one annual booster that can be administered to all age groups. Transmission-blocking vaccines were also included in this development effort. Importantly, the Roadmap prioritized access to low cost GMP vaccine manufacture for commercial production, which encompasses a direct pathway to licensure, ability to manufacture at a large scale, as well as vaccine availability and ease of access, inclusive of delivery platforms and adjuvants. With these considerations in mind, we review enabling vaccine delivery platforms and adjuvants with favorable attributes, to both facilitate the fusion of promising malaria targets with novel technology platforms and meet Roadmap vaccine development, efficacy, and accessibility goals.

A brief overview of recent findings regarding development of immune responses during and after malaria infection is relevant to vaccine development (particularly regarding parasite immune evasion mechanisms that engage inflammatory pathways, promote T cell exhaustion, and stimulate regulatory T cell expansion), as vaccination and malaria infection occur concatenately in endemic regions. Traditionally, the intended outcome of any combination of malaria vaccine target and adjuvant/delivery platform would be to elicit as strong of an immunostimulatory response as possible to the Th1 and/or Th2 immune compartments. However, studies of chronic disease in humans and mice, in the cancer and infectious disease fields, have shown that magnitude of the immune stimulus may not be as important as the balance of immune presentation to immune overreaction (e.g., regulatory T cell stimulation and T cell exhaustion/ablation) [3–5]. Recent studies in rodent malaria models have significantly increased our understanding regarding how chronic malaria infection can hamper development of effective Th1 and Th2 immune responses as well as development of B and T cell memory. For example, in a mouse model of severe malaria infection, it has been shown that proinflammatory cytokines (IFN-γ and TNF) and pathways mediating the disease are detrimental to development of humoral response by inhibiting/exhausting T helper cells [6]. That these cytokines are the same as those monitored in regards to enhanced cellular response to many adjuvanted vaccines is of particular concern. Furthermore, in mouse malaria models, chronic malaria infection triggers CD8+ T cell exhaustion (loss of T cell effector function) through a programmed cell death-1 (PD-1) pathway [7, 8]. In humans, the state of T cell exhaustion has been documented following numerous infections, during chronic infection, as well as in cancer patients, with expression of PD-1 as a hallmark exhaustion [9]. Taken together, these phenomena suggest an explanation for some of the difficulties encountered during malaria vaccine development as well as provide insight into why vaccine-induced protective immunity quickly wanes. A knowledge base on preventing T cell exhaustion has been developed in the therapeutic cancer vaccine field, which can be used as a launching point for how to address this issue in malaria vaccine development. One of the key areas of interest is the use of adjuvants to downregulate pathways leading to T cell exhaustion (e.g., PD-1 and LAG-3 inhibitors) [10–12]. Therefore, we suggest that the information presented herein be considered in the context of careful characterization of the mode of action for the combined antigen and adjuvant/delivery technology to overcome the evolving hallmarks of malaria immune evasion.

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1.1. Enabling vaccine delivery platforms

A range of novel vaccine delivery platforms are described herein, including those that can accommodate different antigen/immunogen formats such as recombinant proteins, peptides, epitopes, and/or nuclei acids. Many of these platforms can deliver multiple antigens and in different formats, simultaneously. In describing these platforms, we included state-of-the-art technologies with the potential for major impacts to vaccine delivery where the regulatory and/or licensure pathways may not yet be defined. As there are a significant number of published preclinical and clinical studies on different viral vectored malaria antigens (e.g., adenovirus and modified vaccinia Ankara (MVA) vectors), these platforms have not been detailed herein, but have been recently reviewed elsewhere [13–15]. In addition, several particulate-based platforms that have been used in combination with malaria candidate vaccines, but not described here, have been recently reviewed [16].

1.2. Virus-like particles

The ability of viral capsid proteins to self-assemble and incorporate foreign antigens has been exploited in the development of virus-like particles (VLPs) as a vaccine delivery platform [17, 18]. While many VLP platforms for human pathogens have been developed and utilized to deliver native epitopes of the subject virus, other VLP platforms can be used to deliver foreign epitopes; only the latter is discussed herein. Platforms capable of integrating foreign epitopes include human pathogens (e.g., hepatitis B virus (HBV) and human papillomavirus) and many nonhuman pathogens (e.g., bacteriophages, plant viruses, and animal viruses). Foreign antigens can be incorporated into VLPs by genetic modification of the capsid protein at one or more sites or by chemical conjugation of antigen to the capsid protein. Immunogens can also be encapsidated within the VLP lumen. Even with these modifications, capsid proteins can retain the structure and morphology of the originating virus. VLPs are known to induce strong humoral and cellular immune responses as foreign antigens are displayed in a repetitive manner on VLPs, which boost immune responses by facilitating cross-linking of immunoglobulin receptors and B cell activation; VLPs are easily taken up by antigen-presenting cells that ultimately lead to cytokine production, stimulation of CD4+ T helper cells, and induction of potent cytotoxic immune responses through cross-presentation to cytotoxic CD8+ T cells [18–24]. For most VLPs, single-stranded RNA (ssRNA) is packaged within the particles during assembly. The presence of ssRNA is thought to enhance immunity, as this is a natural ligand for Toll-like receptor (TLR) 7 and TLR8 [25]. Of note is that the described plant-based VLP platforms do not meet a strict definition of VLP as they are infectious to and propagate in the organism in which they are grown (but not in humans). For simplicity, and as these platforms are often identified as VLPs in the literature, they are presented together in a single section.

The main drawback of VLP-based vaccines is size constraints of the foreign antigen incorporated or fused to capsid protein, which is dependent on the specific VLP platform. Note that larger antigens can be chemically conjugated to some capsid proteins; however, this is also a drawback as the antigen and capsid must be expressed and purified separately for conjugation, which can elevate cost. Currently, three VLP-based malaria vaccine candidates have been

evaluated in the clinic, and one of these was recently approved for use in humans. These are RTS,S (tradename Mosquirix when in combination with AS01), ICC-1132, and Pfs25 VLP-FhCMB, which are each described below. A recent review of the different GlaskoSmithKline (GSK) adjuvants (AS01, AS02, etc.) can be found elsewhere [26].

1.2.1. Hepatitis virus VLPs

Viruses of the family *Hepadnaviridae*, including the human pathogen HBV, can be utilized as vaccine delivery platforms. These viral particles have both an outer lipid envelope containing a surface antigen (e.g., HBsAg) as well as a nucleocapsid composed of a core protein (e.g., HBcAg). Both the surface protein and core protein can accept foreign peptides and thus be utilized as VLP-based vaccine delivery platforms. RTS,S is composed of the repeat and C-terminal regions of *P. falciparum* circumsporozoite protein (PfCSP) fused to hepatitis B virus surface antigen (HBsAg) and has been licensed in combination with AS01 by the European Medicines Agency (EMA). The vaccine candidate ICC-1132 is comprised of the hepatitis B virus core protein (HBcAg) with PfCSP T cell and B cell epitope insertions. ICC-1132 has been tested in multiple clinical studies [27–30]; however, upon controlled human malaria challenge (CHMI) of vaccinees administered with ICC-1132 formulated in Montanide, no sterile protection was seen [30].

Core protein-based VLPs possess a number of favorable characteristics for vaccine development as compared to surface protein-based VLPs. These include the ability of the recombinant core protein to self-assemble into VLPs, flexibility of the expression system, and increased immunogenicity of the core protein as compared to the surface protein [31–34]. Considerations of immune tolerance issues when using VLPs based on the human pathogen HBV are also important given the number of chronic HBV carriers worldwide. Additionally, as antibodies to the HBcAg serve as the basis for HBV diagnostics, widespread use of HBcAg-based VLPs may compromise use of anti-HBc antibodies to diagnose infection. These concerns have been addressed through use of nonhuman pathogenic hepadnaviruses in VLP platform development, including viruses that infect rodents and ducks [35–37]. In addition to RTS,S and ICC-1132, which are VLPs based on the human hepatitis B virus, a woodchuck hepatitis B virus VLP platform (WHcAg) containing PfCSP T cell and B cell epitopes (developed by VLP Biotech) has shown promise in challenge studies with a rodent malaria model where 80–100% protection was seen with different formulations [38]. In this same study, a WHcAg VLP carrying *P. vivax* circumsporozoite protein (PvCSP) repeat epitopes was used for challenge with a rodent malaria model and 100% protection was seen. A comparative study of antigenicity and immunogenicity of different rodent hepatitis virus core proteins (woodchuck, ground squirrel, and artic squirrel) and HBcAg demonstrated that rodent core proteins are (1) equal in immunogenicity to, or more immunogenic than HBcAg for both B cell and T cell responses, (2) not significantly cross-reactive with the HBcAg for B cell responses and only partially cross-reactive with HBcAg for T cell (CD4) responses, and (3) competent to function as vaccine carrier platforms for heterologous, B cell epitopes [36, 39]. In consideration of cost, WHcAg VLPs can be easily expressed at high levels in *E. coli*. In addition, this platform can

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accommodate insertion of foreign sequences at multiple sites within the coat protein and large inserts at both the N- and C-termini. Note that this platform has not yet been tested in the clinic.

1.2.2. Bacteriophage VLPs

VLPs based on ssRNA bacteriophages can be used as vaccine delivery platforms through conjugation of foreign antigens to the coat protein, encapsidation of foreign antigens within the VLP, or genetic insertion of foreign sequences into the coat protein. One application of the last is creation of VLP-based peptide display libraries by the University of New Mexico (based on MS2, PP7, and AP205 bacteriophage VLP platforms) using a series of plasmid vectors that allow insertion of high complexity random sequence peptides into the coat protein enabling construction of libraries with up to 10^{10} to 10^{11} unique random sequence peptide VLP clones with inserts from 6 to 20 base pairs in length [40, 41]. Biopanning can be performed on these libraries using neutralizing or inhibitory antibodies to affinity select VLPs containing mimotopes—peptide sequences that mimics the structure of epitopes. As opposed to traditional peptide display technologies, affinity selected bacteriophage VLPs can be used directly as immunogen without modification. For example, a VLP mimotope to reticulocyte-binding protein homologue 5 (RH5) found through affinity selection of MS2 libraries using a monoclonal antibody able to block parasite invasion of erythrocytes (*in vitro* via growth inhibition assay) was shown to elicit inhibitory antibodies when administered to mice as an immunogen [42]. In addition, MS2 VLP library affinity selection has also been performed with two anti-AMA-1 monoclonal antibodies [42, 43]. Of note is that Agilvax LLC (a startup that was spun out of the Science & Technology Corporation at the University of New Mexico) holds an exclusive license to commercialize this technology for vaccines and immunotherapies based on the MS2 and AP205 VLP platforms. The MS2 VLP platform can also be used to encapsidate RNA and RNA-modified cargo [44]. Similar to the Qbeta VLPs, foreign antigen can be conjugated to AP205 VLPs [45]. Additionally, while most bacteriophage VLPs can only tolerate small foreign insertions, relatively large insertions are tolerated by the AP205 platform [46]. This has been exploited to create a platform whereby antigens can be irreversibly bound to and displayed on AP205 VLPs by simple mixing using SpyCatcher and SpyTag; further, AP205 VLPs utilizing the SpyCatcher/SpyTag system and displaying Pfs25 (a transmission-blocking vaccine target) are immunogenic in mice [47, 48]. Note that use of the SpyCatcher/SpyTag technology might easily be extended to several other platforms included here, in particular to nanoparticles and lipid vesicle-based platforms; however, it will be important to understand if this technology can meet regulatory requirements for clinical use.

1.2.3. Plant-based virus VLPs

A number of plant viruses have been developed into replication-competent platforms whereby VLPs can infect and replicate in plants (generally tobacco or spinach), but not in humans. Two of the more advanced platforms include alfalfa mosaic virus (AlMV) and tobacco mosaic virus (TVM), both of which can be produced at high levels in plants [49]. While the benefits of biologics production in plants include the ability to produce large quantities of material with relatively low cost starting materials, there are logistical challenges in housing and transfecting

plants on a large-scale under conditions that meet GMP requirements as well as development of downstream purification processes to recover VLPs from fibrous plant material [50]. Of note is that significant improvements have been made in plant cell culture for expression of pharmaceutical products (conducted in bioreactors), which has the potential to alleviate some of these challenges and increase the feasibility of using plant VLPs platforms for large-scale vaccine production [50, 51]. Malaria vaccine research efforts have included development of a AlMV VLP containing Pfs25 by Fraunhofer USA Center for Molecular Biotechnology [52], which has been tested in a phase I clinical trial (ClinicalTrials.gov Identifier: NCT02013687); however, results of the trial have not yet been published.

1.3. Polymeric nanoparticle delivery platforms

Polymeric nanoparticles share many of the immunological advantages of VLPs in that their size and structure are similar to that of a pathogen, which leads to interaction with antigen presenting cells [53]. An additional attribute of polymeric nanoparticles is that these can be tailored to a specific purpose through adjusting physical attributes such as size, shape, and charge as well as customizing the type and concentration of polymer [54]. A number of different nanoparticle-based vaccine delivery platforms are available, some of which have been used in the clinic and some of which have thus far only been assessed in animals. The main components of these platforms are biodegradable polymers/composites that function to create a depot for antigen presentation. Immunostimulators can also be incorporated into these delivery platforms. Antigens may be encapsulated or bounded by covalent or noncovalent bonds.

1.3.1. PLGA nanoparticle delivery platforms

Poly-DL-lactide-co-glycolide acid (PLGA) is a biodegradable polymer approved for human use by FDA for several indications. Published applications of PLGA nanoparticles in regards to malaria vaccine development include the targets Pfs25 and PvCSP [55, 56]. PLGA nanoparticle delivery platforms of interest for malaria vaccine development include Selecta Biosciences' synthetic vaccine particle (SVP™) platform and Orbis Biosciences Stratum™ platform. The SVP™ platform in combination with TLR7/8 and TLR9 agonists can enable robust cellular and humoral immune responses, and is flexible regarding how antigens are incorporated [57]. The Stratum™ technology is based on PLGA microspheres that encapsulate aqueous material (including antigens) and degrades in a controlled manner to permit delayed release of encapsulated material [58, 59]. For example, this platform can enable provision of both prime and booster antigen within a single administration with the booster contained in extended release microspheres that degrade 30 days after administration. Clinical studies have been conducted on PLGA-based platforms from both Selecta and Orbis.

1.3.2. Multilayer nanofilm-based nanoparticles

Artificial Cell Technologies, Inc. (ACT) has developed an innovative method for producing nanoparticle vaccines utilizing artificial biofilms comprised of oppositely charged polymers and target antigens constructed on solid $CaCO_3$ cores (nonimmunogenic carrier) using layer-

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by-layer (LbL) fabrication. This platform can accommodate multiple target antigens applied at different layers (depths) within the biofilms as well as immunostimulators, as needed for optimizing the immune response. Additionally, the biolayers can include innate immune stimulants to increase vaccine potency. Biofilm generation and LbL fabrication of nanoparticles have been previously described [60–62]. Of note is that experimental studies have shown that proteins/polypeptides are stabilized in nanofilms by noncovalent interactions, and secondary structure is maintained during the manufacturing process [62]. Additionally, disulfide bonds between cysteine-containing peptides increase nanofilm stability, mimicking the stabilization of the native protein structure [62]. Several ACT nanoparticle constructs, including different B and T cell epitopes of PfCSP, have been developed and tested in mice with encouraging results [63]. While this platform has not yet been assessed in the clinic, ACT is fast approaching intuition of a phase I study with this platform.

1.4. Microneedle platforms

Recent advances have increased interest in intradermal/transdermal vaccine delivery due to an improved understanding of the high immune response achievable within the skin, based on plasticity and high numbers of antigen-presenting cells (APCs) within this tissue, including Langerhans cells, dendritic cells, and macrophages [64–66]. Traditional intradermal vaccine delivery, using the Mantoux or skin scarification methods, requires special training to perform correctly and can be inaccurate regarding the dose of vaccine that is delivered or difficult to achieve based on volume limitations (skin scarification). Therefore, platforms that provide easy and accurate transdermal delivery of vaccines are of high interest [67, 68]. Microneedle arrays described herein are solid-state platforms that either integrate vaccine components into a biodegradable polymer, which is subsequently formed into an array, or are manufactured from nonbiodegradable materials into an array and then coated with vaccine components. In either case, size and length of the microneedle array is controlled so that temporary pores are created in the stratum corneum (the protective outer layer of the skin), and the vaccine is administered to the desired depth. In addition to the benefit of "needleless" administration, the depth at which microneedles penetrate does not reach underlying blood vessels or pain receptors. Stabilization of antigens and adjuvants on or within the microneedle array is also of benefit, particularly in regards to cold-chain requirements. One potential drawback of these platforms is that relatively high concentrations of antigen and adjuvant must be possible in order to achieve a relevant dosing range for most vaccines. Also of note is that development costs for microneedle platforms that incorporate antigens are higher than those where antigens are coated on the microarrays. In addition, consideration must be given to the administration device used to place the array regarding cost and ease of use. However, cost savings are achieved with these platforms because a needle and syringe is not required for administration.

Viral vectored ME-TRAP, PbCSP, and PyMSP-1 have been assessed with silicone microneedle arrays in mice where increased immune response to the target antigen (compared to the vector) and protective efficacy were found [69–71]. However, better responses were found using a mixed administration regimen where antigen(s) were first given via the microneedle platform and then boosted via the intradermal route. For these studies, antigen was applied to the skin

just prior to application of the microarray rather than being incorporated into or coated on to the microneedle array itself.

1.4.1. Natural polymeric microneedles

Silk fibroin is a biocompatible, biodegradable block copolymer that self-assembles into β-sheets separated by flexible hydrophilic spacers. This natural polymer is approved by FDA for human use in medical devices such as wound dressings and sutures, and GMP grade silk fibroin is available from Vaxess Technologies. Significant progress has been made by Vaxess in developing a consistent, repeatable manufacturing process for silk fibroin microneedles [72]. Additionally, several protein immobilization strategies can be used with this polymer including adsorption, covalent bonding, entrapment, and encapsulation [73]. One attribute of this platform that is highly attractive for malaria vaccine development is that silk fibroin microneedles can be designed such that an initial bolus of vaccine is delivered upon administration followed by low-level sustained release of vaccine over a period of several weeks or longer [74]. Note that this microneedle platform has not yet been tested in the clinic.

1.4.2. Synthetic polymeric microneedles

Microneedle arrays can also be constructed from synthetic biodegradable polymeric materials such as PLGA. Corium International has a vaccine-in-tip platform where antigen and adjuvant are combined with MicroCor excipients and then the solution is cast into molds to create the microstructure array (MSA) [75]. A PLGA backing layer is applied and the patch integrated into an applicator. The MicroCor platform is designed such that the needles fully dissolve over a period of several minutes after which the backing is removed. Arrays can also be constructed from nonbiodegradable polymers, which are then coated with antigen and adjuvant. Such a platform is available from 3M's drug delivery systems division whereby arrays are molded from medical grade liquid crystalline polymer and substances (antigens, adjuvants, etc.) are coated on the microneedles using a dip coating process [76]. This type of array is designed to be left *in situ* for a short period of time and then removed once the coating has dissolved. Note that liquid crystalline polymer is not biodegradable. Microneedle platforms from both Corium and 3M have been tested in the clinic.

1.5. mRNA-based vaccine delivery platforms

Recent advances in mRNA vaccine delivery have elevated these platforms to the point at which feasibility of mRNA-based vaccines has been demonstrated in the clinic. The CureVac RNActive platform uses mRNA for vaccine delivery and relies on sequence modifications at the 5′ and 3′ ends to enhance protein expression and inclusion of a protamine sequence to increase immunogenicity [77]. An RNActive prostate cancer vaccine has been tested in the clinic with encouraging results regarding safety and immunogenicity, where induction of both Th1 and Th2 responses was seen [78]. There are significant advantages in using a nucleic acid-based platform including (1) the cost benefits of neither having to manufacture/purify antigen nor formulate with adjuvant, (2) the possibility for development of multivalent vaccines without concerns regarding formulation, and (3) quick man-

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ufacturing speeds where gene synthesis to completion of GMP production can take less than 2 months. A parallel mRNA-based delivery platform has been developed by Moderna; however, relatively limited information is available regarding Moderna's mRNA Therapeutics™ platform with the published studies relating to injection of mRNA into the heart (in a mouse model) to treat myocardial infarction [79, 80].

1.6. Densigen™ platform

The Densigen™ platform (available from Altimmune) is based on rationally designed long, fully synthetic peptides (30–40mers) containing natural clusters of CD4+ and CD8+ T cell epitopes (termed densigens). A proprietary bioinformatics approach is applied to select the most immunogenic and conserved domains. Densigens are conjugated to a fluorocarbon moiety, which allows the densigens to self-assemble into micelle-based nanoparticles. The self-adjuvanting properties of densigens are thought to be attributed to persistence of the nanoparticles at the administration site (depot effect) and resistance of the nanoparticles to proteolytic degradation [81]. Multiple densigens can be incorporated into a single formulation. A Phase I clinical study with a densigen-based influenza vaccine demonstrated good immunogenicity to all six peptides contained in the vaccine (across divergent influenza strains) [82]. In addition, a Phase 1 study of an HBV therapeutic densigen vaccine (HepTcell, which is composed of nine densigens) is ongoing (clinicaltrials.gov identifier: NCT02496897).

2. Enabling vaccine adjuvant platforms

Malaria vaccine enabling adjuvant platforms and immunostimulators are detailed in this section. Many of these can be combined with different immunogen formats and vaccine delivery platforms. We describe novel adjuvants as well as those where studies have been performed with malaria vaccine candidates. Also noted is if an adjuvant has been or can be combined with additional immunostimulators. Similar to the vaccine delivery platforms, we included state-of-the-art technologies where the regulatory and/or licensure pathways may not yet be defined.

2.1. Lipid vesicle-based platforms

Several different classes of lipids can be incorporated into vesicle-based vaccine adjuvant and delivery platforms. These include traditional liposomes (phospholipids as well as anionic, neutral, and cationic lipids from bacteria and eukaryotes), lipids derived from viral envelopes (virosomes), and lipids from Archaea (archaeosomes). The versatility and plasticity of lipid vesicle-based platforms are a major advantage as liposomal compositions can be customized to achieve desired characteristics including lipid type(s), charge, size, antigen association type, and inclusion of adjuvants and immunostimulators [83, 84]. Depending on the chemical properties of the liposomes, antigens may be entrapped in the aqueous core, intercalated into the lipid bilayer, and/or attached to the liposome surface by adsorption or conjugation. Additionally, different antigens/adjuvants can be combined to tailor liposomal vaccines for

specific applications [85, 86]. In general, these systems provide adjuvant activity by enhancing antigen delivery to effector cells and/or by potentiating immune responses. Of note is that some of the platforms in this section can serve as both adjuvants as well as delivery platforms, depending on how antigen is incorporated. A comprehensive review of liposome vaccine delivery platforms is provided in [87].

2.1.1. Aqueous liposomal platforms

Several aqueous liposomal platforms have been built around the capabilities of cholesterol to stabilize lipid bilayers and QS-21 to create pores in lipid bilayers through association with cholesterol. In addition to these properties, QS-21 has also been shown to stimulate Th1-type responses and production of antigen-specific cytotoxic T lymphocytes (CTLs) [88]. The molecular stability of QS-21 is increased when incorporated into liposomes [86], as free molecules undergo deacylation above pH 6 and at temperatures problematic for vaccine administration in warm climates where cold-chain may not be maintained [88]. Aqueous liposomal formulations containing QS-21 provided in a format that can be directly mixed with antigen include AS01 from GSK (which also contains monophosphoryl lipid A – MPLA), GLA-LSQ developed by IDRI (which contains a synthetic form of MPLA known as GLA), ALF-Q developed by WRAIR (which contains MPLA), and Matrix-M from Novavax, previously known as AbISCO-100 (which is a unique 40-nm-sized complex and can be combined with immunostimulators such as TLR agonists [89]). These adjuvants are known to promote elicitation of both Th1 and Th2 immune responses [86, 90, 91]. One of the biggest drawbacks for several of these adjuvants is limited access/availability. However, GMP grade MPLA and QS-21 are available from Avanti Polar Lipids, Inc. and Agenus Inc., respectively. In addition, GMP grade lipids suitable for liposomal preparations are available from Avanti Polar Lipids.

Multiple clinical studies combining malaria vaccines/candidates (RTS,S, LSA-1, AMA-1, MSP-1, and CelTOS) with this class of adjuvants have been performed [16]. The different levels of protection seen with RTS,S/AS01 are well documented in the literature, and therefore not reviewed here. A high level of protective efficacy has not been found with any of the other malaria antigens combined with AS01, which has been recently reviewed in Ref. [16]. Preclinical studies have been conducted with full length PfCSP recombinant protein adjuvanted with GLA-LSQ where 40% sterile protection was seen upon challenge in a mouse malaria model [92]. This adjuvant has also been used in combination with PfCelTOS recombinant protein where a statistically significant reduction in liver load was found in challenged mice [93]. In addition, a clinical study with GLA-LSQ and the placental malaria vaccine candidate PAMVAC (VAR2CSA) has been registered on clinicaltrials.gov (identifier: NCT02647489); however, no published information is available. ALF-Q is a relative newcomer, and there are currently no published studies using ALF-Q in combination with malaria vaccine candidates. Matrix-M has been tested in preclinical studies with a variety of antigens including viral-vectored Pv Duffy binding protein (DBP) vaccine candidates. High levels of *in vitro* erythrocyte binding inhibition (>90%) were achieved with Matrix-M formulations in this study [94]. Additionally, Matrix-M has been assessed in a Phase I clinical study in combination with chimpanzee adenovirus 63

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(ChAd63) ME-TRAP and MVA ME-TRAP (ClinicalTrials.gov identifier: NCT01669512); however, study results have not yet been published.

A platform related to Matrix-M and also comprised of lipids, cholesterol, and QS21 is immune stimulating complex (ISCOM) technology. ISCOMs are spherical open cage-like structures formed by cholesterol binding QS-21 and then stabilized with phospholipids [95]. Similar to Matrix-M, ISCOMs have a mean diameter of ~40 nm; however, this technology incorporates antigens into the ISCOM such that they are displayed in a multimeric fashion on the particles. ISCOMs are known to traffic antigen into the cytosol of dendritic cells, stimulate both Th1 and Th2 immune responses, and link innate and adaptive immune responses *in vivo* in a MyD88-dependent manner [96, 97]. The major drawback of this platform is that the type and amount of antigen displayed can be limiting; however, researchers have developed additional methods for incorporating antigen into ISCOMs, including using cationic ISCOMATRIX particles to attract anionic proteins, adding a lipid tail to the antigen, or using a fusion protein strategy to add hydrophobic peptide tags to the antigen [98]. Although modification of a vaccine candidate for inclusion in such a platform can add a layer of complexity and additional expense to the development process, ISCOMs might be of interest to adjuvant malaria immunogens containing a GPI-anchor. No studies with ISCOMs and GPI-anchored proteins were found in the literature. ISCOMs are commercially available from CSL Behring as ISCOMATRIX.

Another aqueous liposomal platform of potential interest is the adjuvant CAF01, developed by Statens Serum Institute. A clinical study conducted with this adjuvant demonstrated long-lived Th1 responses over a 3-year monitoring period (ClinicalTrials.gov identifier: NCT00922363) [99]. This adjuvant is comprised of two synthetic components, cationic liposomes and a glycolipid immunomodulator (synthetic mycobacterial cell wall cord factor). In a recent preclinical mouse study, five approved or clinically tested adjuvants (Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared head-to-head [100]. Of the five adjuvants tested, CAF01 was the only adjuvant to elicit a Th1 immune response when formulated with each of the three antigens. In addition, the CAF01 formulation demonstrated the highest reduction of *M. tuberculosis* and *Chlamydia trachomatis* in challenge models. However, in most cases, the humoral response elicited was similar to the no adjuvant and/or alum formulations. Note that MF59®, GLA-SE, and IC31® are discussed in subsequent sections.

2.1.2. Lipid-in-oil platforms

The DepoVax™ platform, developed by ImmunoVaccine Inc., contains lipids, cholesterol, oil, emulsifier, and an immunostimulant (e.g., polyIC or Pam_3Cys) [101]. This unique lipid-in-oil platform is designed to present antigen(s) and adjuvant(s) at a long lasting depot that effectively attracts APCs and from which antigen is released over an extended period of time, from weeks to months [102]. Based on this long lasting depot, a single dose of vaccine formulated in DepoVax has been shown to be superior to multiple doses of the same vaccine formulated in traditional adjuvants [103, 104]. In addition, DepoVax has been shown to not only promote Th2 responses, but also enhance Th1 immune responses without triggering

regulatory T cell [101]. DepoVax has been used in the clinic as part of a Phase I/II study for a cancer vaccine (ClinicalTrials.gov identifier: NCT01095848) [105]. Of note is that there are no aqueous components in this formulation; therefore, antigen is lyophilized for use with DepoVax and components are mixed and emulsified prior to administration using materials provided as part of an administration kit.

2.1.3. Virosomes

Virosomes are liposomes prepared by reconstituting virus envelope phospholipids. Those from influenza virus are the most common with the virosome physicochemical properties modulated by the amount and type of lipids used [106]. In contrast to liposomes, virosomes can contain functional viral glycoproteins (i.e., influenza virus hemagglutinin and neuraminidase) within the phospholipid bilayer membrane, which enhance immunogenicity. Additionally, virosomes can induce both B and T cell responses through antigen presentation in the context of both MHC-I and MHC-II [87, 106, 107]. Antigen can be encapsulated within the virosomes, conjugated to phospholipids, or adsorbed to the virosome surface [108]. Malaria antigens have been incorporated into influenza virosomes including epitopes from AMA-1, CSP, MSP-3, and GLURP [109–111]. Clinical studies were performed with virosomes containing an AMA-1 peptide from domain 3 (PEV301) and a CSP repeat region peptide constituting constrained NPNA units (PEV302). In a Phase 1a trial, volunteers immunized with PEV301, PEV302, or a combination of the two (PEV301 + PEV 302) had good seroconversion and long-lived humoral responses when assessed at a 1-year follow-up [112–114]. A Phase 1b trial was conducted in a malaria-endemic area where adults and children were immunized with PEV3B (a formulation including both the subject AMA-1 and CSP epitopes) or a comparator virosome-based vaccine to influenza, Inflexal®V [115]. While not statistically significant, the malaria incidence rate in children administered PEV3B was lower than children given Inflexal®V (67% versus 80%, respectively) over the 1-year follow-up period.

2.1.4. Archaeosomes

Species of the domain *Archaea* contain unique polar lipids that have adjuvanting properties when used as liposomes (archaeosomes) containing encapsulated antigens. The lipid backbones found in *Archaea* have a higher resistance to acid hydrolysis compared to those from *Eukarya* and *Bacteria*, and surface tension and permeability characteristics of archaeosomes differ in comparison to traditional liposomes; these properties are also thought to increase adjuvanting potential of archaeosomes [116]. The type of immune response generated with archaeosomes can be manipulated by changing composition of the head groups attached to archaeol, and lipids from some *Archaea* have been used to chemically synthesize additional lipid types with interesting characteristics [117, 118]. Several studies with archaeosomes and encapsulated ovalbumin (for cancer applications) have shown elicitation of both Th1 and Th2 immune responses as well as the ability to modulate the immune response by varying the types of polar lipids within the archaeosomes [117–119]. As this is a relatively new platform, a pathway to regulatory approval for clinical testing and licensure is needed.

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2.2. Polymeric microparticle-based adjuvants

Advax™ (developed by Vaxine) is a microparticle-based adjuvant comprised of microcrystalline delta inulin, a plant-derived polysaccharide. Of note is that delta inulin is insoluble at body temperature. This adjuvant generates Th1 and Th2 immune responses and activates the alternative compliment pathway [120]. However, Advax™ does not activate nuclear factor-kappa B (NFkB) so an inflammatory response is not seen with this adjuvant [121]. Given the mounting data regarding the ability of malaria to hinder development of effective immune responses, adjuvants that do not trigger inflammatory responses are of interest, particularly for use in malaria-endemic regions. Although there are no published studies with Advax™ and malaria antigens, preclinical assessments have been conducted with a variety vaccine candidates formulated in this adjuvant [121]. In addition, this adjuvant has been assessed in multiple clinical studies [122, 123].

2.3. Emulsion-based adjuvants

All of the adjuvants described in this section are oil-in-water emulsions. Those that include solvents and surfactants form nanoemulsions, which facilitate antigen uptake by dendritic cells [124]. Formulation of malaria vaccines with emulsion-based adjuvants is of interest in part because there are examples of a high level of sterile protection in clinical studies with CHMI challenge when RTS,S was combined with an oil-in-water emulsion-based adjuvant [125, 126]. The GSK adjuvants AS02 and AS03 are both oil-in-water emulsions. AS03 contains squalene, vitamin E, and Tween 80, while AS02 contains these components plus MPLA and QS21. In a clinical study with CHMI where RTS,S was combined with either AS02 or AS03, sterile protection was seen in 6/7 (86%) volunteers given RTS,S/AS02, and 2/7 (29%) volunteers given RTS,S/AS03 [126]. However, 6 months after the last vaccination only 1/5 (20%) of the volunteers given RTS,S/AS02 showed sterile protection upon a second CHMI [125]. For these volunteers, the factors contributing to loss of protective efficacy in such a short period of time is unclear but may relate to challenge and the ability of malaria parasites to compromise development of long-term immunity. In light of these and similar results, improvements in sustaining established immunity must be made regarding development of an effective malaria vaccine.

2.3.1. MF59

MF59 is an oil-in-water nanoemulsion that consists of squalene oil, Tween 80, and sorbitan trioleate (Span 85). MF59 (available from Novartis) is licensed in Europe as a clinical vaccine adjuvant for influenza and has been intramuscularly administered to millions of people ranging in age from children to elderly adults. This adjuvant has been assessed in animal studies with MSP-1 and PvDBP malaria antigens; however, immunogenicity and binding assay (PvDBP) results were poor in comparison to other adjuvants [127–129]. While these results were discouraging, using MF59 in combination with small molecule immunostimulators, particularly those known to induce a Th1 response such as CpG (which has been previously used MF59 with encouraging results [130]), may improve efficacy with malaria antigens. In a recent study, five approved or clinically tested adjuvants

(Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared in head-to-head mouse studies [100]. Of the five adjuvants tested, only MF59 and GLA-SE were able to induce inhibitory titers to influenza; however, inhibitory titers with MF59 were >60% higher than with GLA-SE. Note that MF59 formulations did not induce a Th1 immune response for any of the antigens.

2.3.2. NanoStat™ platform

NanoStat™ (available from NanoBio Corporation) is an oil-in-water nanoemulsion composed of soybean oil, ethanol, Tween 80, cetylpyridinium chloride, and water. Note that these components are quite inexpensive as compared to those of some of the other adjuvants described herein. The adjuvant activity of this nanoemulsion is dependent on the nanodroplet structure and positive charge, which enables the penetration of the mucous layer, binding to cell membranes, and cellular uptake [124, 131]. In mice, NanoStat™ has been shown to produce systemic and mucosal immune responses including MyD88-independent Ab responses and MyD88-dependent Th-1 and Th-17 cell-mediated responses [132]. While most of the published research (including a Phase I clinical [133]) is with NanoStat™ formulated for intranasal delivery, a formulation that contains the same components in proportions tailored for intramuscular administration is also available. These adjuvants can be combined with small molecule immunostimulators. Currently, there are no published studies of NanoStat™ used in combination with malaria antigens.

2.3.3. GLA-SE

GLA-SE (developed by IDRI) is an oil-in-water emulsion containing squalene and glucopyranosyl lipid A (GLA), a synthetic Toll-like receptor 4 (TLR4) agonist that is similar to MPLA. This adjuvant is known to generate both Th1 and Th2 immune responses. Multiple clinical studies have been conducted with GLA-SE formulated vaccines [134–136] including several with malaria antigens; however, results from the latter have not yet been published. Preclinical studies with GLA-SE have been conducted with a number of malaria vaccine candidates. As an example, in a study conducted with a full length PfCSP recombinant protein (produced by Pfenex) adjuvanted with GLA-SE, 50% sterile protection was seen upon challenge in a mouse malaria model (Pb/PfCSP repeats and C-terminal region replacement) [92]. With a different full length PfCSP recombinant protein (produced by WRAIR), 60% protection was seen upon challenge in a different mouse malaria model (Pb/PfCSP full-length replacement) [137]. This adjuvant has also been used in combination with PfCelTOS recombinant protein in preclinical studies, where a statistically significant reduction in liver load was found in challenged mice [93]. A clinical study with PfCelTOS formulated in GLA-SE has been conducted, but data have not yet been published (ClinicalTrials.gov identifier: NCT01540474). Additionally, in a preclinical study with PvRII (region II of PvDBP) where moderate levels of in vitro erythrocyte binding inhibition (>50%) was achieved with GLA-SE, slightly higher levels (>60%) were seen when GLA-SE was combined with the TLR 7/8 agonist R848 [138].

In a recent study, five approved or clinically tested adjuvants (Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared in head-to-head mouse studies [100]. Of the five adjuvants tested, only GLA-SE demonstrated statistically significant inhibition in all three challenge models. However, it was not the best performer in any of the models.

2.3.4. Nutritive immune-enhancing delivery system (NIDS)

NIDS (developed by Epitogenesis, Inc.) is an oil-in-water nanoemulsion containing vitamin A, a polyphenol-flavonoid, catechin hydrate, Tween 80, and mustard oil that were originally developed to boost mucosal immune responses to a variety of antigens without triggering inflammatory responses [139]. All the adjuvant components are generally regarded as safe (GRAS) by the FDA and are available at GMP-grade. Note that these components are quite inexpensive as compared to those of some of the other adjuvants described herein. The NIDS platform allows modification of the NIDS components toward more Th1 or Th2 responses [140]. In addition, this adjuvant can be administered systemically (e.g., IM injection) or delivered via a mucosal route. Although this adjuvant has not yet be assessed in the clinic and no preclinical studies have been published that use NIDS in combination with malaria antigens, it is worth consideration regarding both its low cost and ability to adjuvant in the absence of triggering an inflammatory response.

2.4. Small molecule adjuvants and immunostimulants

Several small molecule-based adjuvants and immunostimulators relevant to malaria vaccine development are described herein. Note that although a number of TLR agonists have been used in preclinical (and in some cases clinical) assessments of malaria vaccine candidates, these have been thoroughly reviewed elsewhere [141, 142]. Such TLR agonists include MPLA/GLA (TLR4 agonists), CpG oligodeoxynucleotides (ssDNA containing cytosines and guanines, which are TLR9 agonists), Poly:IC (dsRNA, which is a TLR3 agonist), Pam3Cys (lipopeptide and TLR1/2 agonist), as well as imiquimod and resiquimod (TLR 7/8 agonists), none of which are detailed herein.

2.4.1. IC31® adjuvant platform

IC31® (developed by Valneva) is a two-component adjuvant comprised of a polycationic peptide (poly-L-arginine) and ODN1a (a TLR9 agonist). Activity of this adjuvant includes recruitment of MHC class II at the injection site as well as migration of antigen to the draining lymph node [26]. The poly-L-arginine contributes to development of humoral and Th2 immune responses [143], while ODN1a is a single-stranded DNA oligonucleotide that stimulates Th1 responses [144]. No studies of malaria vaccine candidates formulated IC31® have been published. In a recent study, five approved or clinically tested adjuvants (Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared in head-to-head mouse studies [100]. Of the five adjuvants tested, IC31® elicited Th1 and Th2 immune responses and demonstrated a statistically significant

reduction (but not the biggest reduction) of *M. tuberculosis* and *C. trachomatis* in challenge models. This adjuvant has been assessed in the clinic [145–149].

2.4.2. 7DW8-5

7DW8-5 (developed by ADARC) is a glycolipid α-galactosylceramide (α-GalCer) analog identified as part of structure-activity relationship (SAR) screening of α-GalCer analogs for increased adjuvant activity as compared to the parent molecule [150]. 7DW8-5 induces Th1 immune responses by binding CD1d (nonclassical MHC proteins expressed on APCs that present lipid antigens), stimulating natural killer cells, and inducing dendritic cell activation/maturation as well as dendritic cell trafficking to the draining lymph node [150, 151]. This adjuvant has been assessed in preclinical studies with several malaria vaccine candidates including CSP, AMA-1, and irradiated sporozoites [151–153]. In formulations with 7DW8-5, enhanced Th1 responses were found as well significantly reduced liver load [153] and a high level of sterile protection (90%) upon challenge [151].

2.4.3. Saponins (QS-21)

Saponins (particularly QS-21), which can be extracted from the bark the *Quillaja saponaria* Molina tree or be semisynthetic, are of interest because they have been shown to stimulate Th1-type responses and production of antigen-specific cytotoxic T lymphocytes (CTLs) [88]. However, a major concern regarding use of QS-21 in malaria vaccines is instability at pH above six and at elevated temperatures [88]. While it is possible to stabilize QS-21 (e.g., in lipids with cholesterol), this small molecule is not recommended for malaria vaccines formulations (intended for use in endemic regions) without assessments of stability at elevated temperatures in the selected formulation.

3. Conclusions

In recent years, there has been a proliferation of novel and promising adjuvant and vaccine delivery systems that together cast a wide net over the effector targets offered by our current understanding of immune system's pathways. Accordingly, this review surveys the field of vaccine delivery platforms and adjuvants in the context of their potential utility for improving the sterile or protective immunity conferred by malaria vaccine candidates. These technologies have the potential to positively affect the induction of immune response elicited by a vaccine candidate through more effective antigen delivery and presentation, ability to present multiple epitopes/copies of epitopes, and mobilizing different components of the immune system appropriate to the antigen and the malarial life-cycle stage being targeted.

Indeed, the judicious selection of vaccine delivery platforms and adjuvants is a necessary part of the malaria vaccine development process. For example, RTS,S was tested with a range of different adjuvants in both preclinical and clinical studies prior to section of AS01 for the commercial formulation. Additionally, while many other malaria vaccine targets have since been tested with AS01, none of these has achieved the same level of protective efficacy seen

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with RTS,S/AS01 [16]. These data demonstrate the necessity of evaluating malaria vaccine targets with a range of delivery platforms and adjuvants prior to selection of the platform(s)/adjuvant(s) for clinical testing. In addition, testing multiple formulations will likely be necessary to sufficiently evaluate efficacy. In endemic regions, this process is compounded by the parasite's ability to hamper development of effective, long-lived immune responses as the intersection of vaccination and infection varies greatly depending on the level of malaria transmission. Several strategies for addressing these complications have been presented including, (1) platforms capable of sustained antigen release, (2) adjuvants that function without triggering inflammatory immune responses, and (3) use of blockage inhibitors to reduce T cell exhaustion.

Additional considerations include affordability and cold-chain requirements. As the principal target populations for vaccination against malaria are individuals residing in endemic regions, primarily Africa and other developing countries, an effective vaccine must be relatively inexpensive to manufacture, store, and deliver. Several of the enabling technologies presented are relatively inexpensive. Additionally, some have the potential to stabilize the vaccine formulation for room temperature storage and transport. However, accessibility is problematic for several of these technologies, and there is a significant cost component regarding the development process, particularly for the vaccine delivery platforms. Due to the complex nature of malaria vaccine development, it is clear that a team approach capable of tapping into expertise in the commercial, academic, government, and nonprofit sectors to efficiently assemble the right combination of vaccine development and delivery technologies is critical to success of malaria vaccine development strategies.

Acknowledgements

This publication was made possible through support provided by the Office of Health, Infectious Diseases, and Nutrition; Bureau for Global Health, U.S. Agency for International Development, under the terms of the Malaria Vaccine Development Program (MVDP) Contract AID-OAA-C-15-00071, for which Leidos, Inc. is the prime contractor. The opinions expressed herein are those of the author(s) and do not necessarily reflect the views of the U.S. Agency for International Development. The authors would like to thank Carter Diggs and Lorraine Soisson for helpful discussions regarding the adjuvants and vaccine delivery platforms described herein.

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Section 5

Entomology and Vector Control

Chapter 18

Exploiting the Potential of Integrated Vector Management for Combating Malaria in Africa

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65467

Abstract

Integrated Vector Management (IVM) is advocated by the World Health Organization (WHO) as the pivotal platform for vector control. The threat for malaria and emerging and re-emerging vector borne diseases is increasing. However, adoption and deployment of the IVM strategy has been minimal. Though malaria endemic countries are embracing and consolidating the IVM approach, real time entomological data on transmission risk and targeting the right vector with the appropriate intervention is lacking. IVM could be harnessed for circumventing operational constraints for vector control. Herein IVM for combating malaria and other insect-borne diseases is reviewed and ways to maximize its potential and benefits are proposed. IVM promotes operational research for evidence-based, cost-effective and optimally sustainable vector control with judicious integration of available options, improves management of insecticides, and effective mitigation of potential negative health and environmental impacts. IVM enhances institutional arrangements including accountability, collaboration and coordination of stakeholders. IVM will require policies and frameworks to maximize intervention impact; and infrastructure and human resources capacity, community involvement and information sharing, strengthened regulation for registration and quality assurance, procurement, financial management and supply chain management for commodities. However, national health system-based response among stakeholders and political commitment is needed for optimal IVM implementation.

Keywords: integrated vector management, malaria transmitting mosquitoes, insect-borne diseases, strategic planning, operational frameworks, evidence-informed decisions, intersectoral collaboration, capacity building

1. Introduction

Malaria remains the biggest health threat among vector-borne diseases (VBDs) with approximately 214 million annual cases and a related 438,000 deaths occurring worldwide in 2015, particularly in sub-Saharan Africa [1, 2]. Though vector control is a proven approach for controlling and eliminating disease, its continued efficacy is likely to be compromised by a multiplicity of constraints [3]. To mitigate the problem, endemic countries in collaboration with the World Health Organization (WHO) have implemented various vector control tools over the years. The quest by WHO for rational evidence-based vector control approaches can be traced through several conceptual stages as: integrated vector control strategy, 'utilization of all appropriate, safe and compatible means of control to bring about an effective degree of vector suppression in a cost-effective manner' [4]; selective vector control, 'application of targeted, site specific and cost-effective activities to reduce disease morbidity and mortality' [5]; and comprehensive vector control, 'control of the vectors of two or more co-prevalent diseases through a unified managerial structure using similar or different vector control methods' [6]. Until recently, management of some vector control programmes have mostly been vertical in disposition. To maintain the effectiveness of vector control, the integrated vector management (IVM) strategy was established and advocated by the WHO as a pivotal and recommended platform for combating VBDs [7]. As such, vector control programmes in endemic countries are encouraged to adopt, establish and implement national policies for the approach. By definition IVM is 'a rational decision-making process for optimal use of resources for vector control' [8]. The IVM strategy is based on the premise that effective control is not the sole preserve of the health sector but of various public and private agencies, including communities. It uses sound principles of management and allows full consideration of the determinants of disease transmission and control [8].

Effective IVM policies incorporate available health resources and infrastructure, uses methods based on knowledge of factors influencing local vector biology; disease transmission and morbidity, deploys a multi-disease approach, integrates recommended environmental, biological or chemical interventions, often in combination and synergistically, includes inter-sectoral collaboration within the health sector and with other public and private sectors that impact on vectors; engagement of local communities and other stakeholders; and a public health regulatory and legislative framework , and reinforces vector control management systems [7]. To circumvent vector control-related constraints in the face of dwindling public-sector human and financial resources, the WHO provides technical assistance [8] and documents, guidance on policy-making [9], structure for training [10], handbook for IVM [11] and monitoring and evaluation [12] to facilitate implementation processes. Unfortunately, only a limited number of countries have harnessed IVM to operationalize efficacious VBD control. The IVM approach has five key strategic elements: advocacy, social mobilization and legislation; collaboration within the health sector and with other sectors; integrated approach; evidence-based decision-making; and capacity building [7]. Notably, an IVM-based process should be underpinned by operational research and monitoring and evaluation of impact on vectors and disease transmission, cost-effective, encompassing requisite infrastructure, financial resources and adequate human resources to

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manage and implement vector control [13]. Nevertheless, substantial challenges for implementation exist at national and local levels and operational experience for the strategy is still limited to relatively few countries.

While vector control has potential to control and eliminate VBDs [2], it has been invariably compromised by: the emergency of insecticide resistance in disease vectors coupled with the lack of sustainable financial resources to sustain control programmes [7], scarcity of personnel with requisite skills and minimal or lack of collaboration between health and other relevant sectors (e.g. infrastructure development, agriculture, environment and educational sectors) to effectively monitor and manage it [5]. Other constraints include residual and outdoor malaria transmission, nominal real-time entomological and epidemiological information to understand transmission risk and to target the right vector with the right control method at the right time; environmental, socio-cultural, socio-economic, technical and programmatic setbacks; weak health systems; limited access to health services; lack of pesticide and resistance management plans; weak planning and coordination amongst disease control programmes. Equally, population increase, returnees, internally displaced and nomadic behaviour of people preclude effective deployment of interventions, particularly in emergencies. The situation is further aggravated by the absence of guidance on IVM in humanitarian emergencies and exclusion of emergency situations in the WHO Handbook for IVM [11].

The need for strengthened human and infrastructural resources to improve vector control has been a topical issue deliberated upon at various forums [14]. Accordingly, appeals and proposals to address inherent challenges have invariably been made to bilateral and multilateral organizations, funding and implementing partners [15]. Nevertheless, limitations that are operationally crucial and with potential to compromise effective programming locally still remain minimally addressed. Though highly divergent at country levels, in the face of shrinking resources, the need to adequately circumvent these pertinent shortcomings is obvious and inevitable. Well-implemented IVM provides a potential platform that could be exploited for enhanced entomological monitoring and surveillance, strategic insecticide resistance management (IRM) planning and rational VBD control and elimination of transmission [16]. In this chapter, an attempt is made to critically review and appraise information on malaria vectors and other insect-borne diseases that could potentially be controlled in tandem, using available tools and management strategies for their control and bringing to the fore some of the pertinent challenges experienced in operational settings. A framework of policies and strategies to facilitate the implementation of the IVM approach is presented and will accentuate coordinated responses amongst stakeholders and political commitment for effective policy execution within the context of national health systems.

2. Methods and literature search strategy

The need to streamline vector control efforts for mosquito-borne diseases, particularly malaria is the case for this review describing a 'literature overview' about implementation of IVM for combating diseases. Information sources for this review included all available data and

accessible archived documentary records on malaria vector control. Structured literature searches of published, peer-reviewed sources using online scientific bibliographic databases were utilized to gather pertinent data from endemic countries. This was conducted via systematic literature search of Library catalogues and online electronic databases, particularly PubMed [17], WHO Library Database [18], Google Scholar [19], African Journals Online [20], Armed Forces Pest Management Board [21], and research for life database HINARI [22] were used to search for the relevant literature. All digital electronic database searches for peer-reviewed, published work used a combination of key search terms: (1) complex of *Anopheles* malaria vectors and one of the following terms; (1) 'IRS' and IVM; (2) ITNs/LLINs and IVM; (3) LSM and 'vector control' and 'prevention' and 'surveillance'; (4) malaria epidemiology; (1) and (4); (2) and (4); and (3) and (4); vector control, epidemiology, malaria, house improvement, *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus*, biological control for malaria control and innovative malaria vector control tools, dengue fever, chikungunya, yellow fever, lymphatic filariasis, Western equine encephalomyelitis, St. Louis encephalitis virus (SLE), human African trypanosomiasis, South American trypanosomiasis (Chagas disease), leishmaniasis, onchocerciasis, Zika and trachoma. Literature was also reviewed from settings with similar framework strengthening approaches. Reference sections of all relevant articles were also reviewed to identify more literature. Additional non-peer reviewed literature including strategic and implementation plans and annual reports were examined for information related to the subject. The inclusion criteria considered all manuscripts and publications in English language that report on malaria vector control and IVM. The literature was reviewed and applicable research findings and key concepts from other countries in different regions were considered for inclusion and translation into this framework.

3. Important insect-borne diseases and responsible vectors

The threat of malaria [14] and emerging and re-emerging VBDs such as dengue fever [23], chikungunya [24], yellow fever [25] and lymphatic filariasis [26], most of which are mosquito-borne, is increasing. Vector control need assessments for IVM conducted in several countries indicate the presence of multiple VBDs with divergent endemicities and spatial distribution. Globally, there are many important vectors of malaria parasites such as *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles funestus* in Africa; *Anopheles stephensi* and *Anopheles culicifacies* in Asia; *Anopheles albimanus* and *Anopheles darlingi* in the New World tropics; and *A. labranciae* and *Anopheles sacharovi* in Europe. Detailed descriptions of the updated global distribution of malaria vectors are provided elsewhere [27, 28]. Many species of *Aedes* and *Culex* are vectors of arboviruses that infect various vertebrates, including humans. *Aedes aegypti* is a principal urban vector of the yellow fever virus and is the primary vector of the dengue viruses. *Aedes albopictus* is a primary vector of forest yellow fever and a secondary vector for dengue fever in most regions. Chikungunya vectored by *Ae. albopictus* and *Ae. aegypti* has increasingly been reported [24]. *Culex quinquefasciatus* is a vector of the nematode worms causing lymphatic filariasis (LF) and of several arboviruses. LF is also transmitted by *An. gambiae*, *An. arabiensis* and *An. funestus* mosquitoes in several countries. *Culex pipiens* is the

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vector of Tahyna virus in Europe, St. Louis encephalitis virus (SLE) in North America, and West Nile virus in several continents. *Culex tritaeniorhynchus* is the primary vector of Japanese encephalitis virus in Asia, and *Culex tarsalis* is the primary vector of Western equine encephalomyelitis and SLE in western North America. *Aedes* (*Neomalaniconion*) *mcintoshi* and related species are important enzootic transmitters of Rift Valley fever virus in Africa [29]. Human African trypanosomiasis is vectored by *Glossina fuscipes*, *Glossina tachinoides*, *Glossina palpalis*, *Glossina pallidipes* and *Glossina morsitans* species of tsetse flies. South American trypanosomiasis (Chagas disease) vectored by *Triatoma infestans*, *Panstrongylus megistus*, *Rhodnius prolixus*, *Triatoma brasiliensis* and *Triatoma dimidiata*. Leishmaniasis (kala-azar) is transmitted by *Phlebotomus orientalis*, *P. argentipes*, *P. papatasi*, *P. longipes*, *P. sergenti*, *P. martini* and *P. celiae* species of sand flies. Onchocerciasis (River Blindness) is vectored by *Simulium damnosum* complex and *Simulium neavei* group black flies in Africa, and *Simulium ochraceum*, *Simulium metallicum* and *Simulium exiguum* complexes in Central and South America. Other commonly endemic diseases include trachoma vectored by *Musca sorbens* bazaar flies [30]. Recently, outbreaks of yellow fever were reported in Ethiopia, South Sudan [31], Zambia [25], DRC and Angola [32]. Notably, the global scale of insect-borne diseases is enormous despite most of them having World Health Assembly resolutions passed on combating them [32, 33].

4. Epidemiological context of malaria

The global burden of malaria remains threateningly high, especially in Sub-Saharan Africa where the disease is endemic in most areas and the region is responsible for about 90% of all malaria deaths globally [2]. The majority of people reside in areas with relatively stable malaria transmission with few inhabiting areas supporting seasonal and less predictable transmission, because of either altitude or rainfall patterns. In areas of stable malaria transmission, the disease burden is highest amongst very young children and pregnant women within the population. An estimated one million people in Africa die from malaria each year and most of these are children under 5-year old [34, 35]. The consensus view of empirical studies, reviews and well informed expert opinion is that malaria causes at least 20% of all deaths in children under 5 years of age in Africa [36]. In most areas, malaria transmission is high, defined as greater than one case per 1000 residents and perennial with substantial seasonal variation in intensity [37]. *An. gambiae* s.s., *An. funestus* and *An. arabiensis* are the main vectors with marked temporal and spatial distribution variation at certain times of the year [38]. Vectorial capacity is high due to abundant rainfall (>2000 mm/annum), elevated year-round temperatures and high humidity [39]. Approximately 98% of malaria cases are due to *Plasmodium falciparum* and is responsible for all severe forms of the disease and deaths [2].

In 1998, the Roll Back Malaria (RBM) partnership revived global malaria control efforts [34]. By 2007, these efforts were invigorated following a call for global malaria eradication [35, 40]. While substantial global progress has been made with 34 countries advancing towards elimination, 64 countries remain in the control phase, mainly in Africa where malaria remains endemic [2]. Examples of countries that successfully eliminated malaria include: Australia in 1960, Taiwan in 1965, Mauritius in 1998, Morocco in 2002 and the United Arab Emirates in 2007

[41]. Between 2000 and 2010, malaria mortality rates fell by 26% globally and by 33% in the World Health Organization (WHO) African Region with approximately 1.1 million malaria deaths averted [2]. Pivotal to these gains, principally in Africa, has been the expansive scale-up of insecticide-based interventions against malaria vectors [42]. From 2008 to 2012, USD 5.83 billion was spent for malaria control in Africa by international donors, and endemic countries scaled up WHO proven interventions including vector control [2]. Generally, attempts to control the anopheline vectors have been limited and intermittent and have had little apparent impact on the huge overall malaria burden [43]. However, the epidemiological trend of malaria disease in the WHO African region has changed significantly over the past years, particularly, due to improved vector control using indoor residual spraying (IRS), long-lasting insecticidal nets (LLINs) and larval source management (LSM), in addition to effective treatment with *artemether-lumefantrine* [43]. There has been a sustained impact on malaria disease burden, evidenced through the reduction in the morbidity and mortality in Namibia, Swaziland, Botswana and Eritrea thus paving the way for them to potentially achieve elimination [44]. However, progresses made in the last 50 years in restricting the geographical areas affected by malaria are being eroded recently, due to changes in land use, global climate changes, armed conflicts/movement of refugees, international travel and emergency of insecticide-resistant vectors.

Vector control is the only available intervention able to reduce transmission at the inception stages of malaria elimination and plays a pivotal role during the attack phase [41]. Vector control remains vital in knocking out the remaining foci of transmission during the later stages of elimination; post-elimination, its role is reducing outbreak risk and as a defence against reinvasion [41, 45]. However, transmission-reducing interventions must be cognizant of behavioural and bionomic attributes of the local vector species including the geography and epidemiology of the malaria foci to be attacked. Therefore, the formulation of plans of action for vector control activities within the malaria elimination strategy should take this information into consideration [45, 46]. The attack phase will aim at maximum intensity and complete coverage throughout to interrupt transmission entirely using selective IRS and universal coverage with LLINs for utmost impact [41, 47]. Nevertheless, there is need to guard against potential challenges such as technical and operational obstacles, procurement, forest malaria, human resource, sustainability, limited data on vector bionomics, insecticide resistance in malaria vectors and gradual declines in both the technical quality of spraying operations and acceptance by target communities. In this regard, capacity building will be critical for successful delivery and effectiveness of the vector control programme [41, 47]. Currently, the global technical strategy for malaria is prioritizing vector control and surveillance. New strategies for vector control are emphasizing IVM [48].

As countries pursue malaria elimination, with strong indications that many, will achieve their goal, IVM approaches will be critical. In countries with well- established IVM, intensive health promotional campaigns and provision of necessary materials have resulted in active participation of communities in vector control activities [44, 49]. To best achieve set goals, an integrated approach in controlling the diseases is recommended wherever feasible given the endemicity overlaps and transmission similarities of some VBDs. This requires adherence to

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all the five key strategic elements of the approach. Recognizing that the IVM approach has been adopted and implemented as the main platform for vector control that include deployment of IRS and LLINs supplemented by LSM [49, 50], there is need for countries to set out to update, revise and widen the scope of their IVM strategies, because of increasing malaria transmission and challenges in vector control. However, the most useful and feasible options for IVM to be applied to the IVM frameworks are: capacity building, integration across diseases, different sectors and communities, evidence-based approach, and collaboration within the health sector and other sectors [42]. This is critical and on effective and efficient deployment of existing and new vector control interventions, to generate and share evidence on integration of all vector control tools and to work with all RBM partners to build entomology and vector control capacity in endemic countries.

5. Rational for integrated vector management

More than half of the world's population is at risk of VBDs, and over one billion people are infected and more than one million die from these diseases annually [1, 2]. In tropical and subtropical regions, climate is most favourable to major VBDs. High diversity of vector-species complexes has the potential to redistribute themselves to new climate or intervention-driven habitats leading to new disease patterns [3]. Up to now, effective reduction of VBDs burden has primarily been ascribed to well-planned and implemented vector control [48], nevertheless, its full potential benefits have not been exhaustively exploited due to limited capacity to manage and deploy vector control; nominal collaboration between infrastructure development and the health sectors; and the detrimental effects of insecticide resistance in disease vectors [51]. To this effect, a Global Strategic Framework setting out the principles and approaches to IVM was developed by WHO [7, 48]. Though IVM forms the cornerstone of contemporary efforts for the prevention and control of VBDs, the approach has only been operationally harnessed in settings with relatively well-established health systems [44, 49]. Only 62% of 113 endemic countries globally and 53% of countries in Africa have national IVM policies and implemented the strategy [52]. IVM has been prompted in part by limited knowledge about deployment in post-conflict settings, where delivery structures are lacking or less developed; very little efforts towards integrating control of multiple VBDs and use of various tools with vector control targeting a single disease and are not fully integrated into health systems, thus compromising their sustainability.

Despite developing multiple global strategies to combat VBDs with renewed emphasis on vector control, high rates of morbidity and mortality are still ascribed to malaria [2, 7]. Vector control need assessments have demonstrated a variety of key factors that undermine the effectiveness of vector control, including: sub-optimal choice or improper timing of interventions and subsequent waste of valuable resources resulting from inadequate capacity for evidence-based decision-making to guide vector control strategies at national, regional, district and community levels; the effect of climate change, environmental degradation and urbanization on malaria and other VBDs, necessitating an adaptive management approach to vector control underpinned by local evidence; lack of collaboration and coordination with other

pertinent sectors such as agriculture, industrial works and construction including communities, culminating in limited awareness of the consequences of their actions on the incidence of VBDs; the development of resistance that could potentially undermine effectiveness of insecticide-based vector control efforts [53]. The Stockholm Convention on Persistent Organic Pollutants (POPs) [54] and World Health Assembly resolution WHA50.13 [55] call on countries to design sustainable strategies for vector control. These opportunities, coupled with the presence of arboviral diseases also transmitted by mosquitoes, substantiate the need for an IVM approach to vector control and the development of guidelines that apply, in principle, to all VBDs but focus mainly on malaria control. The IVM strategic plans would require regular adaptation to changes in local eco-epidemiological or socio-economic conditions. IVM provides a unique opportunity to develop cross-disease control programmes, thus facilitating generation and establishment of entomological and epidemiological baseline data for future tracking of performance of interventions. The IVM also plays a pivotal role in mitigating the impact of resistance on vector control interventions as part of resistance management approaches and their inherent effect on control tools [53].

6. Malaria vector control interventions

The arsenal for insecticide-based contemporary malaria vector control is unnervingly limited to a small number of insecticide classes. Furthermore, extensive exposure of malaria vectors to insecticides eventually selects for resistance to them. This necessitates thorough appreciation of available chemical classes, their mode of action, and resistance mechanisms to facilitate utilization of chemical control either in isolation or as part of an IVM approach [56]. Insecticides are classified into four main classes according to their chemical structure. These include chlorinated hydrocarbons (organochlorines) that act by inhibiting the normal functioning of the nervous system. Examples encompass DDT and its analogues that act on the sodium channels of the nerve membrane, benzene hexachloride (BHC) and cyclodienes such as dieldrin acting on the GABA receptors. These insecticides are cheap and easy to manufacture, but their persistence in the environment, wildlife and humans has reduced their use drastically [56]. The second class comprises phosphorothioate insecticides (organophosphates) that include fenitrothion, malathion, pirimiphos methyl and temephos. These insecticides act by binding the enzyme acetylcholinesterase at the nerve junction, and thus commonly called cholinesterase inhibitors. Once bound, this enzyme can no longer remove acetylcholine from the nerve-membrane junction, and the nerve continues to fire in an uncontrolled manner, eventually leading to paralysis and death of the insect. The third class consists of carbamates that have a similar mode of action to that of phosphorothioate insecticides, but they are used in their insecticidally active form as opposed to organophosphates that are invariably administered as the insecticidally inactive phosphorothioate. Examples of carbamates used in vector control are bendiocarb and to a limited extent propoxur. The fourth class is referred to as pyrethroid insecticides. Examples of pyrethroids are deltamethrin, lambda-cyhalothrin and permethrin. Pyrethroids were developed from insecticidally active components of pyrethrum flowers (pyrethrins). These insecticides act in exactly the same way as DDT and its analogues.

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Their lack of persistence makes them good, safe 'knock-down' agents, and they are often still used in aerosols, frequently with the Monooxygenase synergist piperonyl butoxide, which increases their insecticidal activity and reduces their cost. Other insecticides include: insect growth regulators (IGRs) which are compounds that act on the highly species-specific insect hormonal systems (juvenile hormone) that control moulting and metamorphosis. Examples of IGRs are pyriproxyfen and buprofezin; and chitin synthesis inhibitors (benzoylphenylureas) that interfere with the formation of chitin, a major constituent of the exoskeleton of insects. An example of this chemical group is diflubenzuron. The other group involves bio-rationals (bacteria pathogens) that produce toxins capable of disrupting the midgut lining of mosquito and blackfly larvae. The toxins produced by bacteria such as *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus* can be considered as insecticides although they are usually regarded as biological control agents. Detailed descriptions of contemporary and innovative malaria vector control approaches are elaborated elsewhere [57, 58]. A summary is presented below:

6.1. Chemical control

The control of arthropod-borne diseases is anchored by vector control, personal protection and community participation as key WHO strategies. In this regard, vector control aims to reduce and/or interrupt transmission of malaria by preventing human contact with malaria-bearing mosquitoes, reducing the longevity of adult mosquitoes, eliminating breeding sites or killing the mosquito larvae [59]. Contemporary interventions for malaria vector control and elimination encompasses: effective indoor residual spraying; community-wide coverage and use of LLINs; LSM interventions where applicable; and window screens, protective clothing, repellents, etc. The use of IRS and LLINs remains the mainstream malaria vector control tools [60, 61]. Their efficacy has been evaluated widely in different epidemiological settings [62] at community-wide levels [63, 64] and experimental field trials [65, 66]. In reducing abundance and infectivity of malaria vectors, IRS and LLINs reduce overall transmission and confer community-wide protection to all individuals [65], albeit with variation in responsiveness amongst vector populations. Evidence is mounting that combining IRS and insecticide-treated nets (ITNs) affords enhanced protection to exposed populations compared to using one method alone [67]. As such, their deployment together in high malaria risk areas has been advocated [65, 68, 69]. However, the optimal policy for the co-implementation of the two interventions still remains to be determined. Moreover, the development of insecticide resistance in malaria vectors remains a major cause for concern and an increasing threat to these efficacious interventions [70]. Given the inherent diversity in the responsiveness of malaria vectors to control, the core interventions can be supplemented in specific locations by LSM strategies, e.g. larviciding with temephos, biological control and environmental management (EM) in the context of IVM [71].

6.2. Environmental management

The environment plays a particularly important role in determining the distribution of VBDs. Factors that are critical to the survival of different species of disease-carrying vectors include water and temperature, other factors such as humidity, vegetation density, patterns of crop

cultivation and housing. In this regard, managing the environment of the vectors that transmit diseases can be harnessed for controlling VBDs. Environmental management (EM) methods that are particularly applicable to malaria may include destruction of breeding sites by drainage, filling, impounding, or channelling streams and rivers into canals or by altering the vegetation and shade characteristics of the sites favoured by the vectors. However, it should be noted that different species of mosquitoes have distinct larval and pupal water quality requirements. The basic definition of EM for vector control is 'the planning, organization, carrying out, and monitoring of activities for the modification and/or manipulation of environmental factors or their interaction with humans with a view to preventing or minimizing vector propagation and reducing human-vector-pathogen contact' [72–75]. Owing to extreme lack of resources and dependency of vector control programmes on insecticide based interventions, EM has not been widely adopted in Africa, where it could potentially have the greatest impact on vector-borne diseases. However, EM should be part of integrated control programmes devoid of net economic drain on individuals of the community. Notably EM practices have potential to adversely affect the flora and fauna of an area [76], necessitating consideration of environmental impact assessments during the planning stage. Several important species of *Anopheles* that serve as vectors of malaria are able to breed in paddy fields. An environmental control method employed successfully in several countries to control mosquitoes breeding in paddy fields is intermittent irrigation. Intermittent irrigation successfully controlled the malaria vector *Anopheles labranchiae* in rice growing regions of Portugal, the malaria vector *Anopheles sinensis*, and some members of the *An. culicifacies* complex in India [77]. A number of important mosquito species breed in the brackish water of coastal marshes. These include: *Anopheles melas* and *Anopheles merus* in Africa; *An. sacharovi* around the Mediterranean; and *An. albimanus*, *Anopheles aquasalis* and *Anopheles grabhamii* in the Americas [78]. One approach to controlling mosquitoes breeding in salt marshes is to drain the marsh and remove the breeding sites [79]. This method, particularly marsh alteration by ditching, is now largely abandoned for more environmentally friendly methods of control. The other successful approach was exclusion of salt water, which prevented marshes from becoming brackish. Though not universally applicable, this method was effective in controlling *Anopheles sundaicus* in Malaya and *An. sacharovi* in Italy [80]. Some mosquito species, such as *An. albimanus*, can breed in fresh water as well as in brackish water, and the existence of fresh water in marshes may provide the water quality required by local fresh water-breeding vectors. All future salt marsh mosquito control programmes based on EM will, therefore, have to be aware not only of the effect that mosquitoes have on people living in and around the salt marsh but also of the impact that any proposed measures will have on salt marsh ecosystems. Environmental methods for vector control in water impoundments, such as the damming of rivers and streams, and the construction of irrigation systems, can dramatically alter the environment both in and around them. In particular, they can provide or enlarge the environment suitable for the breeding of major invertebrate disease vectors. This is exemplified by the Tennessee Valley Authority project that resulted in an outbreak of malaria caused by the vector, *Anopheles quadrimaculatus*, breeding in them [81]. It should be noted that the environmental methods to prevent malaria may also include elimination of breeding sites by drainage or by applying locally grown plants [78].

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6.3. Biological control

Biological control involves the reduction of a target vector population by a predator, pathogen, parasite, competitor or toxin produced by a microorganism. Biological control usually has the advantage, over conventional broad-spectrum insecticides, of target host specificity with corresponding little disruption of non-target organisms in the environment. It is also capable of providing long-term control after a single introduction. The fundamental principles of biological control encompass two aspects: population ecology and mosquito ecology. In population ecology, most populations do not fluctuate around their carrying capacity that the environment can support due to the presence of other mortality factors. Mortality factors are mostly classified as density dependent (DD) or density independent (DI). With DD factors, mortality increases as the population becomes denser and decreases as the population becomes less dense. Density-dependent mortality factors tend to regulate a population around an average size and to resist change to that average size, and are characterized by mortality that is not proportionate to the population density. Therefore, with both DD and DI mortality, biological control will be successful only if the vectorial capacity of the adult vector population declines to an acceptable level [82].

As regards mosquitoes, ecology differs considerably by species, although some generalizations can be made. Mosquito populations are often characterized by rapid increases and precipitous declines. The females are highly fecund, and most species have short generation times under tropical conditions. Thus, most populations can quickly increase when the breeding season begins or rapidly rebound after a catastrophic event. Furthermore, the adults of many species disperse well and the females quickly re-colonize habitats. Larval habitats are diverse, ranging from ephemeral to permanent, from artificial to completely natural, from hoof prints to rice fields, although most species occupy only a well-defined subset of these breeding sites. Different mortality factors predominate in different habitats. DD factors in mosquito populations include predators in permanent ponds of rice fields, overcrowding and competition and, more rarely from predators or pathogens in small-containers, such as tree holes. DI mortality factors such as flooding, temperature extremes and desiccation can be important in any of these habitats. However, the ideal mosquito biological control agent probably does not exist, as it should respond to any vector population increase by rapidly colonizing its habitat and quickly producing numerous progeny. It should be able to efficiently find all vectors, survive periods of mosquito absence, and function well in any habitat. Mosquito natural enemies usually affect the larval stages. The diversity of larval habitats, feeding behaviour and physiology probably provides an insurmountable challenge to the development of a single control agent for all mosquito species [82, 83].

Biological control can be broadly categorized into two divisions: natural biological control which is vector reduction caused by naturally occurring biotic agents, and applied biological control which refers to planned human intervention to deliberately add natural enemies to a habitat to reduce vector population (augmentation) or to protect the agents already present (conservation) by manipulating the environment to optimize natural biological control through minimizing detrimental effects on natural enemies or by enhancing their efficacy. Biological control agents are more likely to cause significant mortality in permanent habitats

than in ephemeral habitats. However, natural biological control alone does not usually reduce vector populations sufficiently to interrupt disease transmission. Two major types of augmentation are inoculation and inundation. With *inoculative* releases, small numbers of natural enemies are introduced that are expected to reproduce in the environment and provide long-term vector suppression over successive generations. For inundative releases, overwhelming numbers of organisms are released to produce an immediate decline in a vector population. Invertebrate pathogens include viruses, bacteria (*Bacillus thuringiensis israelensis* and *B. sphaericus*), protists (*Nosema algerae, Vavraia* spp. and *Amblyospora connecticus*), fungi (*Lagenidium giganteum*, *Coelomomyces* spp., and *Culicinomyces clavisporus*) and nematodes (*Romanomermis culicivorax*). Also, invertebrate predators (*Toxorhynchites* spp. and the copepod *Mesocyclops aspericornis*) and to a lesser extent vertebrates such as larvivorous fish (*Gambusia affinis*, *Poecilia reticulata* and *Aphyocypris chinensis*) have been experimented upon and/or utilized for biological vector control [84]. However, both abiotic (temperature, pH, salinity, organic matter, oxygen levels, toxic agents, habitat type and size and water depth) and biotic (ecology, target species, host specificity and adaptation, dispersal and host-finding abilities, infectivity or capture ability, persistence mechanisms, generation time and population age structure) factors can affect the efficacy and sustainability of biological control, and the suitability of a habitat must be verified at each new location. Biological control has many perceived advantages. It is relatively natural and host specific, it can be self-perpetuating, and it does not adversely affect beneficial invertebrates. As attractive as biological control is in principal, in practice this technique has had limited success in malaria vector control [82, 83].

7. Innovative tools for malaria vector control

Vector suppression plays a pivotal role in the control of mosquito-borne infectious agents. In light of contemporary control constraints, novel approaches are required to achieve effective protection against malaria vectors. Several innovative genetic approaches are being applied to microbial organisms to extend their usefulness as vector control agents. Implementation of IVM requires combinations of control measures that are critical in providing optimal control with minimal detrimental environmental outcomes. With the selection of resistance, new insecticides and novel approaches to vector control are being developed [85]. Innovative approaches invariably being harnessed for contemporary vector control include Durable wall linings, attractive toxic sugar baits, long-lasting topical repellents, spatial repellents, entomopathogenic bacteria traps, fungus-impregnated targets, eave tubes and new molecules for IRS, i.e. chlorfenapyr, including improved housing [42, 86–88].

7.1. Genetic control

Reviews of potential genetic control approaches have invariably included discussion of their operational feasibility against vectors of disease, progress made in population suppression of an increasing number of vectors and population replacement of disease vectors with harmless forms of the same species. Population replacement involves modification of the genetic

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structure of a disease vector in a way that favours human benefit. The desired modification ('useful gene') would be one that prevents disease transmission, either by altering vector behaviour or by producing some physiological change in parasite-vector interaction. The species specificity and non-polluting properties of genetic methods, in contrast to insecticides make the approach attractive. The choice of genetic method for a given species depends on the control strategy to be adopted, whether focal, i.e. adjusted to local needs, or in an area wide rolling programme [89]. The sterile-insect technique (SIT) or sterile-insect release method (SIRM) is the only genetic approach to control that has succeeded on an extensive practical scale with operational effectiveness. SIT is based on the release into the natural habitat of large numbers of the target vector, sterilized in a breeding factory. The released insects carry dominant lethal mutations in their reproductive cells, due to treatment with ionizing radiation or chemical mutagens. All wild female mating with such a male lays sterile eggs. The success of SIT depends on the sterile male's capacity to locate, attract and mate with wild females, in direct competition with wild males. Field trials of SIT against mosquitoes have been conducted on *Culex pipiens quinquefasciatus* in Florida, USA, *An. albimanus* in El Salvador, *An. stephensi* in southern India and *An. arabiensis* in Sudan [89]. The feasibility of integrating sterile-insect technique (SIT), for population reduction of *Ae. albopictus* to prevent and control of chikungunya and dengue, has been considered in the context of IVM in Mauritius [90]. The major technical aspects of mass rearing to be evaluated and improved in mosquitoes are: (1) automated mass rearing, (2) genetic sexing, (3) rapid sterilization, (4) quality control, (5) transportation and release and (6) trapping to evaluate progress [91]. The infection of mosquitoes with *Wolbachia* spp. has been considered to carry a gene for vector competence but would depend on (1) introducing such an 'incompetence' gene into *Wolbachia*, (2) associating *Wolbachia* with *Anopheles* and (3) ensuring that the 'incompetence' gene product, expressed in *Wolbachia*, reaches the gut or salivary glands, which are sites occupied by the malarial parasites [92]. Among all potential methods of genetic control of vectors of disease, SIT has the greatest potential of being effective and economically viable. SIT is at its most effective in combination with other control measures, and it probably extends the effective life of insecticides by slowing down the evolution of resistance [93]. Population replacement remains a distant dream, although research on it provides a rich source of new information on vector physiology and genomics [94, 95].

7.2. Immunological control

Immunological approaches have also been harnessed for the control of malaria vectors. Development of anti-mosquito vaccines reduces blood-feeding and/or pathogen transmission. Their advantages include specificity, safety and cost, ease of administration, long-term protection and absence of residues in the environment. The practicality of vector-blocking vaccines has been given a significant boost by studies that provide protection against pathogen transmission. Advances in immunobiology, genomics, proteomics, DNA immunization and other vaccine-related technologies provide a foundation for new ways to control vectors mosquitoes and malaria [96]. Anti-mosquito vaccines could be designed to disrupt blood feeding, impair reproduction and block development and transmission of mosquito-borne infectious agents. Important resources for anti-mosquito vaccines have been generated from

mosquito expressed sequence tags and genome sequencing projects. Attempts have been made to vaccinate against malaria parasites transmitting mosquitoes such as *An. quadrimaculatus* [97], *An. stephensi* [98–100], *Anopheles tessellates* [100, 101], *Anopheles farauti* [102] and multiple *Anopheles* spp. [103, 104].

Most of these contemporary vector control methods have shown to be successful only in small scale- and, more rarely, in large-scale- and country wide programmes. The IVM strategy provides a platform for integrating these into vector control programmes across the malaria-endemic countries in the tropics. However, clear commitment from national authorities including long-term support from funding partners will be required for effective and sustained malaria vector control [105]. Prompted by fragmentary empirical evidence to inform policy formulation for rational vector control, malaria control programmes are encouraged to adopt the WHO-led IVM strategy [49] underpinned by evidence-based decision-making process and a coherent monitoring and evaluation component [13]. The approach should incorporate routine insecticide resistance surveillance and inherent resistance mechanisms to inform decisions and policy changes on insecticide resistance management operations [106].

8. Status of IVM implementation for combating malaria

Integrated vector management has been harnessed for the control of malaria vectors in various endemic countries according to the five key elements of the strategy. In response to the call by the WHO for member states to implement IVM, most VBD-endemic countries have adopted IVM as a pivotal approach to vector control and developed relevant policy documents particularly for malaria vector control. In Mauritius, IVM is one of the three principal components of preventing reintroduction of malaria. The strategy focuses on bi-weekly routine island-wide larviciding with temephos based on entomological surveillance, and indoor and outdoor residual spraying at and around the port and airport with DDT or lambda-cyhalothrin every six months [90]. IVM targets former malaria foci and high-risk areas around migrant workers' residences [107]. Tanzania has been implementing IVM with ITNs, IRS and LSM including environmental management and larviciding using Bti [108, 109]. Notably, IVM has mostly been harnessed for malaria vector control with minimal utilization as an overarching strategy for VBDs. This is exemplified by Kenya [110], Malawi [15], Mozambique [111], Namibia [44], Tanzania [108], Zambia [49], Zimbabwe [112], most of which were pioneers in implementing the approach. The utilization of IVM for multiple VBD control has been compromised by a lack of requisite resources and the inherent cross-sectoral collaboration. It is noteworthy that in some countries such as Swaziland, Botswana, Lesotho, Namibia, Seychelles, Madagascar and South Africa, the presence of VBDs is almost negligible.

With the increasing emergence (dengue, Zika) and re-emergence (chikungunya, yellow fever) of VBDs, countries have embarked on consolidating their IVM policy and strategic documents. In the Comoros, further to the low levels of insecticide resistance observed in the main

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mosquito vectors of the island (*Cx. p. quinquefasciatus*, *An. gambiae*, *Ae. aegypti and Ae. albopictus*), the optimism is high to implement IVM as a more realistic and feasible approach for the control of malaria, bancroftian filariasis, dengue, chikungunya and rift valley fever [113]. Rwanda has developed a comprehensive IVM strategic plan to improve the ecological soundness, cost-effectiveness and sustainability of vector control interventions. The strategy incorporates interventions with clear implementation and collaborative arrangements, strengthened entomological monitoring and surveillance including resistance management [114]. To improve control of VBDs, Uganda has successfully consolidated strategic planning and operational frameworks for VBD disease control and established an evidence-based IVM approach. The country envisions improved VBD control by operationalizing implementation arrangements as outlined in the IVM strategic guidelines [115, 116]. Equally, South Sudan has developed a draft strategic plan for control of VBDs [117] and has embarked on strengthening frameworks for implementation [51]. Eritrea has updated, revised and widened the scope of its IVM strategy due to increasing malaria transmission and challenges in vector control [118]. Ethiopia has developed an IVM strategy and plan of action to include dengue fever and other VBDs; and establishing a coordination mechanism with relevant sectors, including a multisectoral task force for capacity building in IVM and surveillance; and to actively engage in advocacy, communication and social mobilization.

IVM strategies have been implemented in Mosquito Control Programmes (MCPs), which aim to reduce the cost and optimize protection of the populations against VBDs in the Americas, and research has been conducted to evaluate MCPs strategies to improve vector control activities [119]. Reviews and case studies have been conducted to discuss and analyse the major strategies currently employed in an effort to minimize the burden of VBDs, and the major progress and achievements resulting from international as well as local efforts [120]. Studies considering how one or more interventions can impact multiple VBDs have been conducted to guide the development of an IVM programme to assist the elimination of malaria and lymphatic filariasis [121]. The contribution of vector management methods has also been critically reviewed to prevent and control outbreaks of West Nile virus infection and to present the challenges for Europe [122]. Expansion of IVM to promote healthy environments has been proposed to control pests, improve cleanliness of communities, increase structural soundness and decrease health disparities that arise from external hazards [123].

9. Challenges and opportunities for establishing viable IVM strategies

While the expectation to reduce the burden of VBDs in endemic countries is high, the operational challenges to effective vector control are enormous. Integrated management of malaria vectors still remains an underdeveloped component of malaria control policies in most programmes. This could in part be attributed to the shortage of health workers in general, but other key contributors are technical, operational, political and social economic in nature. Major challenges to malaria control include very high malaria transmission intensity, low coverage of proven malaria control interventions, inadequate health care resources, a weak health

system, inadequate understanding of malaria epidemiology, the impact of control interventions and insecticide resistance development in mosquito vectors. The key challenges are categorized and elaborated further as outlined below:

Limited ability to fully utilize the available tools: In most programmes, vector control is managed by naïve, low calibre and mediocre level public health workers devoid of field experience or is hijacked by clinicians, who are mostly less competent in entomology and vector control. Such programme managers are not receptive to well-informed expert technical advice and are naïve to fully participate in the discourse on vector control and are not open to innovative ideas. The competent public health entomologist and vector control specialists are by and large left frustrated. While surveillance monitoring and evaluation, and operational research are key components of malaria control strategies, activities in the area of vector control are always less prioritized as clinically inclined managers tend to have a predilection for drug efficacy studies. The lack of skills and knowledge has resulted in apathy and unreceptiveness towards scientific results generated by field teams, poor utilization of available tools, low up take of available innovative vectors control tools, vector surveillance, and resistance monitoring and management guidance. While non-governmental organizations (NGOs) play a key role in complementing public sector endeavours in health service delivery, in post-conflict environments, most NGOs have engaged vector control people with minimal or no field experience to effectively transfer competences to the local cadre. Consequently, the IVM strategy has not been fully harnessed for strengthening vector control. However, this trend should be guarded against if meaningful gains are to be attained from contemporary vector control efforts.

Lack or minimal inter-sectoral collaboration amongst stakeholders: Across-sector collaboration amongst stakeholders-public and private sectors—including community empowerment, involvement and participation—and integration amongst VBDs remains an obvert constraint to effective vector control. Even in situations where various partners exist and are working on VBDs control, coordination remains negligible. The situation is aggravated in emergency situations, particularly by the notable absence of entomologists at central level to adequately coordinate entomological resources. There are nominal levels of utilization of available guidance from the WHO and other collaborating stakeholders, as well as limited sharing of experiences by programmes. In recent years, cross-border collaboration and inter-sectoral collaboration on VBDs have been inefficient relative to collaboration on other diseases such as polio elimination [124].

Minimal or absent operational research competences: Efforts to revamp insectaria and entomology laboratories have been embarked upon by various funding organizations. However, entomological infrastructures in most countries are either lying in a state of dilapidation or operating at sub-optimal scale to be useful in any way. They lack requisite equipment and commodities, including a minimal number of dedicated personnel. In certain cases, central laboratories do exist with adequate numbers of staff but lack field laboratory at regional or local levels, making data collection inefficient and thus eroding the potential of existing entomological competencies. There is minimal evidence-based decision-making to provide technical guidance to policy makers and programme managers. Limited

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operational research and country-wide spatio-temporal mapping of VBDs are also amongst the major challenges. The situation is aggravated by inadequate in-country information systems, lack of geo-coding of relevant attributes and centralized data bases for easy information retrieval as and when it is required.

Emergency situations, environmental and climate changes: In conflict and post-conflict environments, security is the major impediment to successful vector control. There is lack of technical and physical infrastructure and personnel are mostly of mediocre caliber. The situation is compounded by high personnel turn over and a lack of touch with the latest information. While these settings experience human resource crises and competent skills are scarce, flexible people who are not possessive about 'the way things are done around here' are needed. Most vector control issues are left in the hands of non-professionals who tend not to be open to constructive criticism. Appropriate requisite regulatory and legislative framework for public health is also non-existent. Equally, the changing climate and land use patterns are having a toll on the efficacy of the once effective vector control interventions. This necessitates innovation and implementation of local situation amenable interventions.

Community sensitization, mobilization and information sharing: There is inadequate and inconsistent social mobilization and community sensitization and insufficient participation and ownership resulting in low compliance with existing interventions. Mass media and interpersonal communication channels have been used to disseminate advocacy, behavioural change and information and education communications to enhance community participation. Unfortunately, such communications never run consistently annually due to limited resources for requisite activities and are hampered by issues of socio-economic and political factors, cultural aspects and alleged abuse of vector control interventions. There is minimal innovative ways to engage the communities in vector control activities including entomological surveillance and resistance monitoring, including information on country and multi-country level experiences with IVM and IRM. Documentation and sharing of lessons learnt from the field will be essential and will to some extent offset the dependency on the overstretched international technical assistance. However, this requires availability of local competences for documenting the experiences.

Funding and operational constraints: Vector control is mostly donor driven, but the funding is often restricted and with prescribed activities. This tends to have detrimental effects on the ambitious and innovative programme officers who may want to adapt the interventions to the local situation. Most development partners have neglected health system strengthening. Therefore, collaborative approaches and strong political commitment and consistent financial support are required to scale up cost-effective interventions for vector control, including focused research and inter-country cooperation and exchange to share best practices. Effective deployment of vector control interventions including entomological monitoring is hinged on efficient operational logistics including transport systems. Programmes in most settings do not have transport dedicated to entomology and vector control but do have pool vehicles, which in most cases are not easy to access as transport officers often lack an understanding of vector control.

10. Recommendations for moving forward

Notably, tackling contemporary vector control challenges will require strategic efforts beyond those aimed at addressing the human resources crisis. Opportunities for strengthening vector control exist and could be harnessed for effective VBD control. Looking forward; the following are proposed priority actions for advancing IVM implementation within the context of its five key strategic elements and the inherent effective monitoring and evaluation in line with the WHO recommendations:

Development of IVM policy frameworks: To improve national vector-borne disease control programmes, countries should develop national IVM policy and national policy on pesticide management. Development of relevant strategic documents and improving the uptake and use of policy documents produced by the WHO will be critical to achieving this end [8]. Particularly in emergency situations that should include the provision of guidance on IVM in humanitarian emergencies.

Institutional arrangements and Inter-sectoral collaboration: Successful inter-country and inter-sectoral collaborations and vector control initiatives are needed to achieve effective control. However, this will require establishing national steering committees on IVM and national coordinating units on vector control, together with developing and field testing approaches for engaging community organizations in an effective partnership with the operational and regulatory personnel of health and local government departments to improve implementation. Although engagement of sectors other than health is important for vector control, the success of inter-sectoral collaboration will be determined by the extent to which other sectors impact vector proliferation [124].

Vector surveillance and insecticide resistance monitoring: Effective IVM strategies need improved entomological surveillance, risk assessment, preparedness and response; including plans for IRM. Therefore, planning and implementation will require the epidemiological surveillance system on vector-borne diseases, and setting up sentinel sites for vector surveillance and insecticide resistance monitoring. In this regard, countries should (1) develop, evaluate, and promote the effective use of vector control methods, in the context of IVM; (2) support improvements in surveillance, including the collection and use of case reports, environmental and demographic data, and other information to better understand disease distribution patterns and strengthen disease control programmes [125].

Operational research agenda on vector-borne disease control: Planning and implementation will require operational research priorities and expected outcomes on vector control to be used in deployment of programmes. In areas prone to malaria epidemics, understanding the local distribution of the disease and its relation to environmental and demographic factors will help public health officials improve the prevention and control activities they direct. The role of operational research and the interactions between research institutions, vector control programmes, civil society organizations, and of financial and technical partners to address challenges and to accelerate translation of research into policies and

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programmes should be considered [14]. Therefore, strengthening the capacity of vector control programmes to conduct operational research, publish its findings and improving linkages between them and research institutes may aid progress towards effective control [126].

Standard operating procedures or protocols need to be developed for entomology teams to collect specimens and map their locations, including insecticide susceptibility status. For the purposes of mapping the presence or absence of a vector, a much more targeted and streamlined sampling strategy with optimal collection methods, e.g. animal-baited traps, light traps, should be determined. Programmes need to plan operational, or implementation research activities for increasing effectiveness of current vector control tools and to better engage specific groups such as the military, mobile and migrant workers and marginalized populations to improve access and appropriated interventions.

Community sensitization, mobilization and information sharing: Advocacy communication and social mobilization would be important in putting national strategic and implementation plans on IVM in place. There should be consistent community sensitization via mass media and interpersonal communication channels to disseminate advocacy, behavioural change and information and education communications and address issues of socio-economic-political factors, cultural aspects, urbanization or climate and abuse of vector control tools and enhance community participation. The key messages, based on scientific evidence and information available and disseminated without delay, are crucial for an effective and urgent response [14]. Campaigns on behavioural change and community mobilization on vector control should be conducted. Lessons learned from programmes will provide the requisite platform for accelerated implementation of IVM.

Capacity building: Developing greater capacity in vector control programmes for collecting, managing and analysing malaria case reports and environmental data is useful for planning control operations. Professional training and experience in entomology and vector control are critical for running an IVM programme that makes appropriate use of all available control methods. Certified training courses on IVM and judicious use of pesticides should be in place at national or regional level. Organization and management of IVM requires standards for professions and careers in vector control and public health entomology to be established [127]. Organizing and implementing regional training programmes, professional associations and technical assistance networks that can operate over the long term to build and sustain the human resource capacity needed for effective vector control programmes. Making headways would require enhancing national vector control capacities in terms of human and infrastructural resources to enable efficient monitoring and management of malaria vectors [128]. Governments in malaria-endemic countries should mobilize enough local resources to supplement the limited and restricted donor funding. As efforts are made towards addressing inherent limitations, seeking rare and often overstretched external technical assistance by control programmes will be critical. This will necessitate institution of local training programmes.

11. Discussion

Most malaria-endemic countries have adopted and implemented the IVM strategy. Although the approach has primarily been harnessed for malaria control, countries are presently consolidating their strategic plans and operational frameworks to incorporate other VBDs in the IVM approach. Efforts are being expended towards establishing requisite infrastructure and human resources for vector control. However, control programmes are still faced with several limitations. Successful IVM would require circumventing contemporary challenges to ensure universal access to interventions.

Effective vector control demands for diligent entomological capacity and necessitate adequate requisite financial and human resources and pertinent physical infrastructural strengthening at national and local levels [7]. The major limitations for capacity-building for IVM are lack of essential physical infrastructure (insectaries, laboratories and equipment), financial resources and technical resources (qualified vector control human resources-medical entomologists and vector control specialists) to support entomological monitoring and evaluation of vector control interventions and to manage and implement IVM. The technical assistance provided by the very few resources that exist is extremely strained. Addressing deficiencies in all these areas of public health capacity would be necessary for the successful implementation of IVM. This would need strengthened collaboration with stake holders including local and international academic and scientific institutions and line ministries such as environment and agriculture.

An IVM-based process should be cost-effective, guided by operational research and subject to routine monitoring and evaluation of impact on vector populations and disease transmission [13]. Evidence-based vector control requires regular collection of comprehensive entomological data from spatially segregated geo-locations to adequately inform decision-making on effective targeting, deployment and monitoring of interventions. Monitoring by countries should incorporate key elements of entomological surveillance, including determining species composition, establishing vector distribution and seasonality, collecting vector behaviour data, conducting insecticide susceptibility testing and establishing underlying mechanisms of resistance. Mosquito vectors have developed insecticide resistance to all four classes of public health insecticides, which are spreading with great potential to compromise vector control efforts [70]. Research on innovative vector control tools should be enhanced to provide adequate convincing evidence that can compel and foster donor flexibility. This will be critical in light of selection of insecticide resistance and outdoor/residual transmission. Otherwise vector control will remain dependent on the archaic tool box full of ineffective interventions. Countries should improve global insecticide resistance tracking and get support for developing management plans and the development of an online interactive platform for mapping level resistance [129].

The WHO recommends IVM as a pivotal platform for combating vector-borne diseases. To this effect, several policy and strategic documents as well as training materials have been developed, including a Global Strategic Framework that sets out the principles and approaches to IVM. For effective vector control, endemic countries need to develop three key documents: (1)

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an IVM-based vector control strategy; (2) a resistance monitoring and management plan; and (3) vector surveillance guidelines, according to WHO recommendations. There is significant opportunity to integrate vector control for VBDs because in many areas either the same vectors transmit more than one disease, or the local vectors share common ecology. Therefore, IVM should focus on redesigning programmes in the context of vector surveillance, insecticide resistance response, environmental and climate change, cross-border initiatives and IVM in emergency situations [129]. The WHO Emergency Response Framework also stresses the need of sufficient risk reduction and preparedness capacities in member states; and institutional readiness of WHO in ensuring that adapted disease surveillance, early warning and response systems are in place. Risk assessments for yellow fever, chikungunya, dengue and Zika should be prioritized in all settings with the presence of *Aedes* spp. mosquitoes.

Successful implementation of IVM requires adequate financial, human and technical resources, a robust entomological monitoring and resistance surveillance and management system, and demands for meticulous operational considerations and preparedness: strengthened operational research to generate data for decision-making and evaluation of new vector control tools; establishing pertinent physical infrastructure-sentinel sites and laboratory and insectary facilities; adherence to WHOPES specifications and WHO recommendations for procurement; institutionalize data repositories and capacity to implement the interventions. Countries should develop strategic and operational frameworks and maximize impact of current tools. This necessitates adequate planning including detailed situation analyses to obtain relevant local data and implementation frameworks (adherence to pertinent regulatory obligations for registration of insecticides, developing overall multi-year budgets for operations). It will need strengthened collaborations with all stakeholders with vested interest in vector control and insecticide resistance including the funders.

12. Conclusion

Despite the slow pace, malaria-endemic counties are embracing and consolidating the IVM approach. The development and implementation of IVM with strict adherence to the five key elements of the approach are crucial for effective operational malaria vector control. Regular need assessments for vector control human and infrastructural resources and technical support to facilitate capacity building in entomological surveillance and monitoring for evidence-based decision-making in vector control are critical for successful IVM, including guiding development of IVM strategic plans, country IRM plans and vector surveillance plans, advocacy for the global plan for IRM (GPIRM), guidance on IVM in humanitarian emergencies, and supporting entomological surveillance, risk assessment, preparedness and response. Effective IVM necessitates strengthening of research, studies of the efficacy and cost-effectiveness of IVM, knowledge, attitude and practice, community participation, and development and harmonization of BCC/IEC messages and effective delivery of materials. Insecticide resistance requires an emergence approach and IVM in humanitarian emergencies need appropriate guidance. The IVM approach can be harnessed as a platform for strategic planning and deployment of vector surveillance, resistance monitoring and management. Thus, rational

IVM strategies should be a pivotal platform for malaria vector control in endemic countries, particularly in conflict and post-conflict or emergency situations. Countries should develop policies, standard operational procedures, country specific guidelines; strengthen human resource capacity, inter-sectoral collaborations, advocacy, legislation and regulation, including community sensitization and engagement. However, significant coordinated response amongst stakeholders and political commitment is needed for timely and effective policy implementation within the context of a national health system. This will require a realization that IVM will yield a positive return for investment in terms of reduced disease burden and the resulting social and economic gains.

Conflict of interests

The author declares that there is no conflict of interests regarding the publication of this manuscript.

Acknowledgements

The author is grateful to the anonymous reviewers for the constructive comments on the manuscript. EC conceptualized this manuscript, formulated the ideas, collected and analysed the data and wrote the manuscript.

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Chapter 19

Resting Behaviour of Deltamethrin-Resistant Malaria Vectors, *Anopheles arabiensis* and *Anopheles coluzzii*, from North Cameroon: Upshots from a Two-Level Ordinary Logit Model

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65463

Abstract

The current study was conducted in Garoua, Pitoa, and Mayo-Oulo health districts of north Cameroon, in order to investigate the resting behaviour of deltamethrin-resistant *Anopheles* (*An*.) *gambiae s.l.* populations and build a model of their response to the use of Permanet 2.0 long-lasting insecticidal nets (LLINs). Adult mosquitoes were collected in October and November 2011, using spray catches and window exit traps in 29 clusters with LLINs in use. Sampled *An. gambiae s.l.* were identified down to species and analysed for blood-meal origin, physiological and circumsporozoite protein status. Deltamethrin resistance was assessed using World Health Organization's (WHO's) standard protocol. A two-level ordinary logit model was used to relate the resting behaviour and deltamethrin resistance. Identified species of the *An. gambiae* complex included *An. arabiensis* (90.6%), *An. coluzzii* (7.1%) and *An. gambiae s.s.* (2.3%). They displayed 1.1–4.8% infection rates, 80% indoor-resting density and 56–80% human blood index. Eleven *An. gambiae s.l.* populations over the 15 tested were resistant to deltamethrin (51–89.5% mortality rates). Model results showed a significant dependence of indoor vector density

on increasing deltamethrin resistance (p-value of <0.01). These behavioural and resistance patterns may lead to increasing malaria transmission in study health districts.

Keywords: north cameroon, long-lasting insecticidal nets, malaria vectors behaviour, insecticide resistance, two-level analysis

1. Introduction

Malaria still remains a priority problem in the world today. According to the latest estimates, there were about 214 million cases of malaria in 2015 (with an uncertainty range of 205–316 million) and an estimated 438,000 deaths (with an uncertainty range of 236,000–635,000) [1]. People living in the poorest countries are the most exposed to malaria, with 90% of deaths occurring in the African region, mostly among children [1]. The emergence of vector resistance to insecticides is a serious threat in the fight against malaria, since the general use of insecticides in indoor residual spraying (IRS) or in long-lasting insecticidal nets (LLINs) constitutes the only means of mass prevention [2]. In many sub-Saharan African countries, vector resistance to all classes of insecticides has been reported [3–5]. Some studies conducted in south western Chad (close to the North Region of Cameroon), where insecticide-treated nets (ITNs) and LLINs are used in large scale, have revealed deltamethrin resistance in the *Anopheles (An.). gambiae s.l.* populations [3, 6]. Given that the successes in the reduction of malaria burden acquired in the long run may strongly be compromised by vector resistance to insecticides, it is necessary to elaborate strategies for the management of this phenomenon.

In Cameroon, malaria is responsible for 30% of morbidity cases, 36% of outpatient consultation, 67% of childhood mortality and 48% of hospital admissions [7]. Malaria infection is essentially due to *Plasmodium falciparum*, followed by *P. ovale and P. malariae* [8]. Seven anopheline species play major roles in *Plasmodium* parasite transmission, among which are *An arabiensis, An. gambiae* and *An. coluzzii*, three sibling species of the *An. gambiae* complex [9]. However, several studies have reported insecticide resistance in these three mosquito species, threatening the progress towards malaria elimination in the country [10–12]. Field observations have shown that the emergence of insecticide resistance is favoured by the abusive and anarchic use of pesticides in public health and/or in agriculture [11, 13]. The regions that are mostly concerned with resistance are the North, Centre, Littoral, West and North West. Two resistance mechanisms have been noticed, *metabolic-* and *kdr*-based insecticide resistance. *Metabolic*-based resistance is due to altered levels of detoxifying enzymes such as P450s, esterases and glutathione S-transferases, whose expression levels may well be modulated by variation in many genes, making it a quantitative genetic trait [14, 15]. The *kdr*-based insecticide resistance results from a single leucine to phenylalanine or serine amino acid mutation at the voltage gate sodium channel which is the target side of dichlorodiphenyltricholoroethane (DDT) and pyrethroid insecticides [5]. *Metabolic*-based resistance is the major mechanism in the northern tropical regions of Cameroon, whereas multiple insecticide resistance, including *metabolic-* and *kdr*-based resistance mechanisms, is widespread in the southern equatorial regions [16–18].

http://dx.doi.org/10.5772/65463

However, while the major mechanisms of insecticide resistance in malaria vectors are more and more documented, an important research gap remains on detailed understanding of the likely genetic basis of specific behavioural traits, and how surveillance programs should be implemented to best monitor changes in these traits. In other words, how insecticide resistance might interact with vector behavioural response to interventions is very poorly known.

The current study was carried out in three health districts in the North of Cameroon, namely Pitoa, Garoua and Mayo-Oulo, where *metabolic*-based resistance to deltamethrin was reported in *An. gambiae* s.s. and the twin species *An. arabiensis*. These species are the major malaria vectors in the North Region, malaria incidence in these districts (46–62%) is among the highest of the 12 health districts of northern Cameroon region [19, 20]. Deltamethrin LLINs (Permanet 2.0) were distributed there at universal coverage in 2010, for malaria mass prevention. The aim of the study was to assess the resting behaviour of local *An. gambiae s.l.* populations when control interventions are put in place, with respect to their insecticide resistance status.

2. Main body

2.1. Experiments

2.1.1. Study area

The field survey was carried out in the North Region of Cameroon from October 2011 to November 2011 in 27 villages in three health districts: Garoua, the regional capital city (9° 30N; 13°40E), Pitoa (9°21N; 13°31E), situated 15 km from Garoua, and Mayo-Oulo (9°46N; 13° 44E), 140 km from Garoua. This is a cotton-cultivated area where there is an extensive use of LLINs for human protection with approximately 87% of households having at least one net and 69% of households having at least one net per two people [21]. The North Region of Cameroon makes up 66,090 km^2 of the Northern half of the Republic of Cameroon with a population density of 13 people/km^2. Neighbouring territories include the Far North Region to the north, the Adamawa Region to the south, Nigeria to the west, Chad to the east and Central African Republic to the south-east. The districts and their study clusters are given in **Figure 1**.

2.1.2. Mosquito collection

Mosquitoes were collected during a cross-sectional survey in 29 clusters including 12 clusters in the Garoua health district, 10 clusters in the Pitoa health district and seven clusters in the Mayo-Oulo health district (**Table 1**). Two separate data sets were collected: one on mosquito's resting behaviour while another was collected on insecticide resistance. For the resting behaviour data, two techniques of adult mosquito collection were used: window exit traps (WETs) and indoor spray catches (ISCs).

WET were placed over the windows of bedrooms to collect mosquitoes that attempt to escape during the night [22]. Ten rooms per cluster were used to collect mosquitoes during two consecutive nights. WETs were placed at 6.00 pm over the windows of each bedroom till 6.00

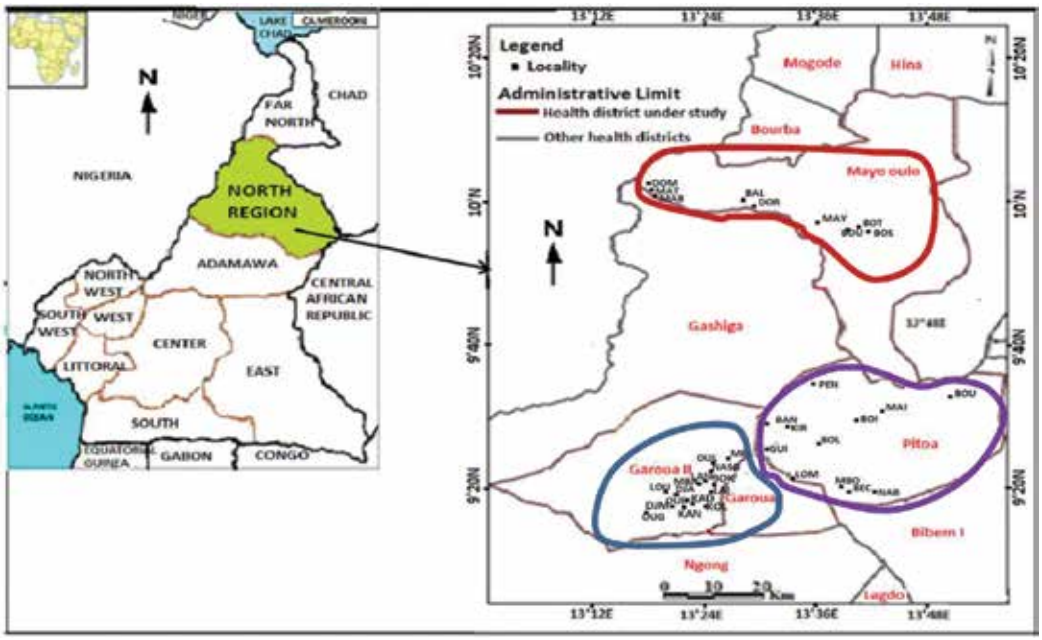

Figure 1. Map of Cameroon showing the study region, health districts and study clusters. The red chart encloses cluster of the Mayo-Oulo Health District (MAY: Mayo-Oulo; BOU: Bouyoum; BOT: Batoum; BOS: Bossoum; DOR: Dourbaye; MAB: Maboni; BAL: Balla). The purple chart encloses cluster of the Pitoa Health District (MAL: Mayo Lebri; BEC: Be Centre; BOI: Boula-Ibbi; BOL: Boulgou; BOUS: Boussa GUI: Guizigaré; NASP: Nassarao; MBO: Mbolom; PEN: Pena KIR; Kirombo). The blue chart encloses cluster of the Garoua Health Districts (DJA: Djamboutou I; DJM: Djamboutou II; KAD: Kanadi II; KAN: Kanadi I; LAN: Laïndé II; LOU: Loundérou; MBA: Mboum-aviation; MBI: Mbilga; BOK: Bocki; NASG: Nassarao; OUL: Oulo Lawane; OUG: Ouro-Garga).

am. Mosquitoes were then collected inside the trap in the morning, between 6 and 8 am using a mouth aspirator.

ISCs were done in the morning, from 6 to 8 pm, to sample mosquitoes resting inside rooms used for WETs. After spreading white sheets to cover the entire floor space and objects, rooms were sprayed with commercial aerosols containing pyrethroids. Mosquitoes fall on to the white sheets 10 min after spraying and were carefully picked up.

Female anophelines collected from WET and ISCs were morphologically identified using identification keys [23, 24]; they were separated according to their physiological status (unfed, freshly fed, half gravid and gravid) and individually preserved in tubes with desiccant for subsequent laboratory analyses.

For insecticide resistance testing, samples of anopheline larvae and pupae were collected from the maximum number of breeding sites. In each cluster, all open water bodies were inspected. Samples of immature anophelines were collected from active breeding sites by the dipping method [22]; they were pooled per cluster and brought to a local insectary, then reared in spring water. Larvae were fed on Tetramin Fish Food (Tetra Werke Company, Inc., Blacksburg, VA, USA) and pupae were kept in a cage to emerge into adults. Emerging adults were

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Health district	Population of district	Selected health areas	Distance to headquarters (km)	Human population	Clusters
		Pitoa	0	32,327	GUI, BOL
		Banaye	13	6296	PEN, KIR
Pitoa	108,611	Be Centre	22	4341	BEC, MBO, NASP
		Bouba Ibib	40	15,262	BOI, MAL, BOUS
Total				**58,226**	
		Kolleré	0	27,692	KAD, KAN
		Nassarao	12	7224	NASG, BOK, MBI
Garoua	316,957	Laïndé	10	56,569	LAN, OUL, MBA
		Djamboutou	7	40,122	DJA, DJM, OUG, LOU
Total				**165,212**	
		Mayo-Oulo	0	28,203	MAY, BOT, BOU
Mayo-Oulo	91,501	Dourbaye	10	16,106	DOR, BAL, MAB
		Doumo	42	8502	BOS
Total				**52,811**	

BAL: Balla; BEC: Be Centre; BOI: Boula-Ibbi; BOK: Bocki; BOL: Boulgou; BOS: Bossoum; BOUS: Boussa; BOU: Bouyoum; BOT: Batoum; DOR: Dourbaye; GUI: Guizigaré; DJA: Djamboutou I; DJM: Djamboutou II; KAD: Kanadi II; KAN: Kanadi I; KIR: Kirombo; LAN: Laïndé II; LOU: Loundérou; MAB: Maboni; MAY: Mayo-Oulo; MBA: Mboum-aviation; MBO: Mbolom; MAL: Mayo Lebri; MBI: Mbilga; NASG: Nassarao; OUL: Oulo Lawane; NASP: Nassarao; OUG: Ouro-Garga; PEN: Pena.

Table 1. Health districts and study clusters in the north Cameroon region.

maintained on 10% sugar solution. Adult anophelines were then morphologically identified as belonging to the *An. gambiae* complex by means of reference keys [23, 24] and used for susceptibility tests.

2.1.3. Insecticide susceptibility test

Insecticide susceptibility tests were performed on mosquitoes aged 2–4 days, emerged from field-collected larvae and pupae. *An. gambiae s.l.* females were exposed for 1 h to 0.05% deltamethrin-impregnated papers using WHO susceptibility test kits and standard protocol for adult mosquitoes [25] under ambient room temperature (25–28°C) and 70–80% relative humidity. Test kits including deltamethrin-impregnated papers, test tubes and accessories were supplied by the Vector Control and Research Unit, University Sains Malaysia (Penang, Malaysia). Filter paper sheets (12 × 15 cm, Whatman N°1) were impregnated with the discriminating dosage of deltamethrin (0.05%) (pyrethroid insecticide), mixed with acetone and silicon oil. Other batches of filter paper sheets were impregnated with acetone + silicon solution for use as control. Acetone acted as the solvent and silicon oil as a carrier. The purchased impregnated papers were stored at 4°C until the date of the test.

Each full set of bioassays was performed with five batches of 20–25 non-blood fed, 2–4-day-old females: four batches were exposed to insecticide-impregnated filter papers and one batch was exposed to untreated filter paper and served as a control. The number of mosquitoes knocked down was recorded at 5-min interval during the 1-h long exposure to deltamethrin-impregnated papers. After exposure to insecticide-impregnated papers or control papers, mosquitoes were transferred to holding tubes and provided with cotton pads soaked with 10% sugar solution. The mortality rates were determined 24 h post exposure.

The resistance status in the mosquito populations was determined according to WHO's criteria [25]:

- A mortality rate in the range of 98–100% indicates susceptibility;
- A mortality rate between 90 and 97% suggests possible resistance to be confirmed;
- A mortality rate less than 90% indicates resistance.

The above criteria are recommended on the grounds that a greater than 2% survival at the diagnostic concentration is considered unlikely to be due to chance alone, and provided that all the test conditions are met.

Tests with mortality of the controls greater than 20% were taken all over again.

2.1.4. Laboratory analysis

The head and thorax of field-collected female *An. gambiae s.l.* were examined for *P. falciparum* circumsporozoite protein (CSP) by enzyme-linked immunosorbent assay (ELISA) [26, 27]. Sporozoite rates were determined as the number of anophelines positive for circumsporozoite protein ÷ the total number of specimens tested by ELISA CSP.

Blood-meal sources were identified by ELISA [28]. The technique identifies human, bovine, ovine (sheep and goat), equine (horse and donkey), pig or chicken hosts. Human blood index (HBI) was determined for each species, that is, the proportion of female anophelines that were found with human blood in their stomachs thereby giving an indication of anthropophilic rate. The HBI was determined as the number of human feeds ÷ (number of human feeds + number of animal feeds).

Genomic DNA was extracted from each selected *An. gambiae s.l.* specimen as described by Collins et al. [29] and each individual was identified to the species level using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [30]. This method allows simultaneous identification of species of the *An. gambiae* complex.

2.2. Statistical analysis

2.2.1. Descriptive analysis

Pareto charts were used for univariate analysis of the qualitative variables *species, district, locality, phy. status* (physiological status), *csp* (circumsporozoite protein status) and *behaviour* (resting behaviour). Box plots were used to see the distribution of the susceptibility variable

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(*loc. suscep*) vis-à-vis the modalities of the qualitative variables and results obtained were tested using the Kruskal-Wallis rank sum test.

2.2.2. Missing data

The *k*-nearest neighbour method of classification with random assignment was used to impute missing observations in the data set, so the missing value in a variable on an individual was imputed by one of its *k*-nearest neighbours picked at random. As a result of this randomness in imputations, constructed models were implemented each on 10 imputed data sets and parameter estimates were obtained for each model by taking the average over the respective 10 imputed data sets estimates [31].

2.2.3. Model specification

A two-level ordinary logit model was used to establish a relationship between *An. gambiae* species caught indoors and at exit traps and the status of deltamethrin resistance in *An. gambiae s.l.* from the same clusters [31]. This model was used because mosquitoes, which were considered as level-1 units, were clustered within localities, considered as level-2 units and measurements were taken at level 1 and 2 units. The model was interested in the effect of resistance on the odds of the probability that a given species *k* in locality *j* will be found indoors against the probability that it will be found at the level of the exit trap. The model therefore has the form: where

$$\begin{aligned}\log\left[\frac{\Pr(\text{behavior}_{jk}=1|x_{jk},z_j,\mu_j)}{1-\text{Prbehavior}_{jk}=1|x_{jk},z_j,\mu_j)}\right] &= \gamma+\beta_{\text{res}}\text{loc.suscep}_j+\beta_{A.gamM}1_{\text{species}_{jk}=A.gamM}+\\ \beta_{A.gamS}1_{\text{species}_{jk}=A.gamS}+\beta_P 1_{csp_{jk}=P}+\beta_{\text{Gravid}}1_{phy.status_{jk}=\text{Gravid}}+\beta_{H.grav}1_{phy.status_{jk}=H.grav}&\\ +\beta_{\text{Unfed}}1_{phy.status_{jk}=\text{Unfed}}+\text{loc.ran.effect}_j,&\end{aligned} \quad (1)$$

- x_{jk} is a realization of the vector of level-1 covariates (*species, csp* and *physiological status*) for a mosquito *k* in locality *j*,
- z_j is a realization of the level-2 covariate (*locality susceptibility*). It is the percentage of deltamethrin susceptibility of locality *j*,
- $\mu_j = \text{loc.ran.effect}_j$ is the locality *j's* random effect in the model assumed to follow a normal distribution with mean 0 and variance σ^2,
- γ, β_{res}, $\beta_{A.gam\ M}$, $\beta_{A.gam\ S}$ β_P, β_{Gravid}, $\beta_{H.grav}$, β_{Unfed} *and* σ^2 are the model parameters to be estimated and
- $1_{X_{jk}=x}=\begin{cases}1 & \text{if} X_{jk}=x\\ 0 & \text{otherwise}\end{cases}$

2.2.4. Estimation of parameters

The maximum marginal likelihood method was used to estimate the parameters of the model. Solutions to the likelihood equations were approximated using Fisher's method of scoring

with numerical approximations of integrals obtained using the Gauss- Hermite quadrature (GHQ) method [31]. Analyses were done in EXCEL and in *R*, using the *R*-function *sabre* in the *R*-package *sabreR* [31–33].

2.3. Results

2.3.1. Vector densities, infection rates and blood-meal origins

A total of 1135 *Anopheline* mosquitoes were collected from October 2011 to November 2011 in 29 clusters across the three study health districts (Garoua, Pitoa and Mayo-Oulo). On the bases of morphological identification, 10 anopheline species were identified in collected mosquito samples, including:

- -three species of the *An. gambiae s.l.* complex representing more than half of the total population of *anopheles* vectors (609/1135= 53.7%),
- -*An. funestus* group (408/1135 = 35.9%),
- -*An. rufipes* (82/1135 = 7.2%),
- -*An. pharoensis, An. nili, An. coustani, An. maculipalpis* and *An. paludis* representing each less than 5% of the total analysed samples.

Among the samples belonging to the *An. gambiae* complex, 299/374 (80%) specimens were successfully identified down to species using PCR-RFLP. *An. arabiensis* was the predominant species (271/299 = 90.6%), followed by *An. coluzzii* (21/299 = 7.1%) and *An. gambiae* s.s. (7/299 = 2.3%) (**Table 2**).

A total of 6/696 anopheline mosquitoes tested by ELISA CSP showed the presence of the circumsporozoite protein, corresponding to 0.86% global infection rate. These specimens belonged to three species: *An. coluzzii, An. arabiensis* and *An. funestus*. The infection rates per

		Physiological status (or gonotrophic state) (%)				
Species	**Point of collection**	**Unfed**	**Freshly fed**	**Half gravid**	**Gravid**	**Total (%)**
An. arabiensis	Indoors	55 (70.5)	99 (84.6)	18 (62.1)	45 (95.7)	217 (80.2)
	Exit traps	23 (29.5)	18 (15.4)	11 (37.9)	2 (4.3)	54 (19.8)
An. coluzzii	Indoors	3 (75.0)	15 (88.2)	0	0	18 (85.7)
	Exit traps	1 (25.0)	2 (11.8)	0	0	3 (14.3)
An. gambiae	Indoors	1 (50.0)	3 (60.0)	0	0	4 (57.1)
	Exit traps	1 (50.0)	2 (40.0)	0	0	3 (42.9)
Total	Indoors	59 (92.2)	117 (84.2)	18 (62.8)	45 (95.7)	239 (79.9)
	Exit traps	25 (7.8)	22 (15.8)	11 (37.9)	2 (4.3)	60 (20.2)

Table 2. Residual and exit trap faunas in relation to species of the *Anopheles gambiae* complex and physiological status. %: proportion of mosquitoes collected indoors versus exit traps.

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species were 4.8% (1/21), 1.1% (3/271) and 0.5% (2/408) for *An. coluzzii, An. arabiensis* and *An. funestus,* respectively.

Four hundred and ninety-seven (497) anophelines were tested for blood-meal origins, among which 229 meals were successfully determined. They included 55.5% (127/229) meals from human, 31.4% (72/229) from cattle and 4.8% (7/229) from sheep and pig (**Table 2**). There were 8.3% (21/229) mixed blood meals from human + pig, human + cattle, human + cattle + sheep, cattle + sheep, cattle + pig and cattle + sheep + pig. The three species of the *An. gambiae* complex (*An. arabiensis*, *An. coluzzii* and *An. gambiae s.s.*) displayed high human blood indexes (HBI), ranging from 0.56 to 0.8, suggesting high anthropophagic tendencies compared with *An. funestus* (HBI = 0.42, $p < 0.005$) (**Table 3**).

Species	H	C	S	P	H/C	H/P	H/C/S	C/S	C/P	C/S/P	Total	HBI
An. arabiensis	94	39	2	2	1	5	1	2	0	0	146	0.69
An. coluzzii	7	3	1	3	2	0	0	0	0	0	16	0.56
An. gambiae	4	1	0	0	0	0	0	0	0	0	5	0.80
An. funestus	22	29	1	2	0	3	1	1	1	2	62	0.42
Total	127	72	4	7	3	8	2	3	1	2	229	0.62
(%)	55.5	31.4	1.7	3.1	1.3	3.5	0.9	1.3	0.4	0.9	100	

H: human; C: cattle; S: sheep; P: pig; H/P: human + pig; H/C: human + cattle; H/C/S: human + cattle + sheep, C/S: cattle + sheep, C/P: cattle + pig; C/S/P: cattle + sheep + pig.

Table 3. Origin of blood meals of the four major malaria vectors in the study health districts.

Figure 2. Mortality rates of *Anopheles gambiae s.l.* populations 24 h post exposure to 0.05% deltamethrin-impregnated papers. The red line at 90% mortality rate indicates the threshold below which a mosquito population is classified as resistant to the insecticide used for susceptibility test [25]. BAL: Balla; BEC: Be Centre; BOK: Bocki; BOS: Bossoum; BOUS: Boussa; DOR: Dourbaye; GUI: Guizigaré; DJM: Djamboutou II; KIR: Kirombo; MAY: Mayo-Oulo; MBA: Mboum-aviation; MBO: Mbolom; MAL: Mayo Lebri; OUG: Ouro-Garga.

2.3.2. Status of deltamethrin resistance in An. gambiae s.l. populations

One thousand two hundred and thirteen (1213) females of the *An. gambiae s.l.* were tested for their susceptibility to deltamethrin. They were from 15 field populations, including six from the Pitoa health district, four from the Mayo-Oulo health district and five from the Garoua health district. Recorded mortality rates are presented in **Figure 2**. According to WHO's criteria, 11 populations among the 15 tested were found resistant to deltamethrin with mortality rates ranging from 51 to 89.5%. Resistance was suspected in three populations, that is, those from Kirambo and Boussa in the Pitoa health district and Mayo-Oulo in the Mayo-Oulo health district, with 92–94% mortality rate. Only the Ouro-Garga population was found susceptible to deltamethrin. These data showed that deltamethrin resistance was widespread in the three study health districts.

2.3.3. Resting behaviour and physiological statuses of the species of the An. gambiae complex

Among the three identified sibling species of the *An. gambiae* complex, high proportions of *An. arabiensis* (217/271 = 80%) and *An. coluzzii* (18/21 = 86%) samples were caught indoors compared with exit traps collection (20 and 14%, respectively), showing their high endophilic behaviour in the study areas (**Table 2**). These proportions were not significantly different between the two species (p-value = 0.25). However, similar rates of *An. gambiae* s.s. specimens were caught indoors versus in exit traps, although the sample size was very small.

Considering the physiological status of collected mosquitoes, all the four gonotrophic states (unfed, freshly fed, half gravid and gravid) were found in *An. arabiensis* samples collected indoors or in exit traps, with freshly fed and unfed mosquitoes being predominant. Meanwhile, only unfed and freshly fed gonotrophic states were found in analysed *An. coluzzii* and *An. gambiae s.s.* samples, raising the question about the behavioural patterns (endophilic/exiting) of half gravid and gravid mosquitoes of these two species.

The overall indoor-resting rate for the three species of the *An. gambiae* complex was 80% (239/299), increasing from half gravid (18/29 = 62%), to freshly fed (117/239 = 84.2%), unfed (59/84 = 92%) and gravid mosquitoes (45/47 = 96%) (**Table 2**). Conversely, the highest rate of exiting mosquitoes was recorded among the half gravid samples (11/29 = 40%) and the lowest among the gravid samples (2/47 = 4%).

2.3.4. Effect of deltamethrin resistance on the resting behaviour

The two level logit regression analysis given in **Table 4** showed a statistically significant dependence of resting behaviour on deltamethrin resistance. Fitting the mean parameter estimates in the model, we obtained the following:

$$log\left[\frac{\Pr(\text{behavior}_{jk}=1|x_{jk},z_j,\mu_j)}{1-\Pr(\text{behavior}_{jk}=1|x_{jk},z_j,\mu_j)}\right] = -14.4626 + 0.1398\text{loc.suscep}_j - 2.36991_{\text{species}_{jk}=A.gamM} \quad (2)$$
$$-2.0974\_1_{phy.status_{jk}=\text{Gravid}} + 1.29431_{phy.status_{jk}=\text{Unfed}} + \text{loc.ran.effect}_j$$

This model showed that the estimated coefficient of the locality resistance variable (*loc.suscep*)

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Variable	Mean estimate of parameter	Variance of imputation method	Standard error estimate	Signal-to-noise ratio	95% C.I. Approximation	Approximated *P*-value
Threshold intercept	−14.463	4.662	2.265	6.385	(−18.902, −0.0232)	0.000
Resistance *loc. suscept*	0.140	0.000701	0.0271	5.158	(0.0866, 0.193)	0.000
Species cies *(An. Arabiensis* = ref) *A. coluzzii* *A. gambiae s.s.*	−2.370 −0.301	0.199 0.711	0.470 0.885	5.0471 0.340	(−3.290, −1.450) (−2.0357, 1.434)	0.000 0.734
csp (Abs = ref) Presence	−16.865	0.265	355.814	0.0474	(−14.259, 680.531)	0.962
Phy.Status (Freshly fed = ref) Gravid Half Gravid Unfed	−2.0974 −0.678 1.294	0.0615 0.185 0.0855	0.264 0.451 0.307	7.936 1.502 4.210	(−2.615, −1.580) (−1.563, 0.207) (0.691, 1.897)	0.000 0.133 0.000
Variance of locality random effect (σ^2)	3.673	0.0864	0.311	11.803	(3.0627, 4.283)	0.000

Table 4. Mean of estimates of parameters in the two-level logit regression model and their corresponding precisions.

	***p*-Value**					
	Behaviour	*Locality*	*District*	*Species*	*Physiological status*	*csp*
Susceptibility to deltamethrin	0.0041	2.2e–16	2.2e–16	0.18	4.1e–06	Observations in same group

Table 5. Kruskal-Wallis rank sum tests of independence between the deltamethrin susceptibility variable and the qualitative variables.

has a positive sign, indicating that the species were more likely to be endophilic (stay indoors) with decreasing values of mortality rates to deltamethrin susceptibility test (increasing deltamethrin resistance). The result was significant at the 5% level (*p*-value = 2.502e–07). This result was confirmed by the Kruskal-Wallis rank sum test (*p*-value = 0.0041) (**Table 5**).

Furthermore, there was varying effect of physiological status on the resting behaviour. **Figure 3** shows the relationships between resting behaviour and deltamethrin resistance for the *An. arabiensis* (A) and *An. coluzzii* species (B) with different physiological statuses.

With specimens of the *An. arabiensis* species, the probability that an unfed specimen was endophilic decreased from 1.00 to 0.31, as the mortality to susceptibility test increased from 50 to 100% **(Figure 3A)**. The total difference in probability was 1.00–0.31 = 0.69. Thus, when the mortality rate was less than 80%, the probability that the unfed *An. arabiensis* was endophilic was greater than 0.88 and when mortality was greater than 98%, the probability was less than 0.38. The effect gradually decreased on freshly fed (total difference in probability was 1.00 −

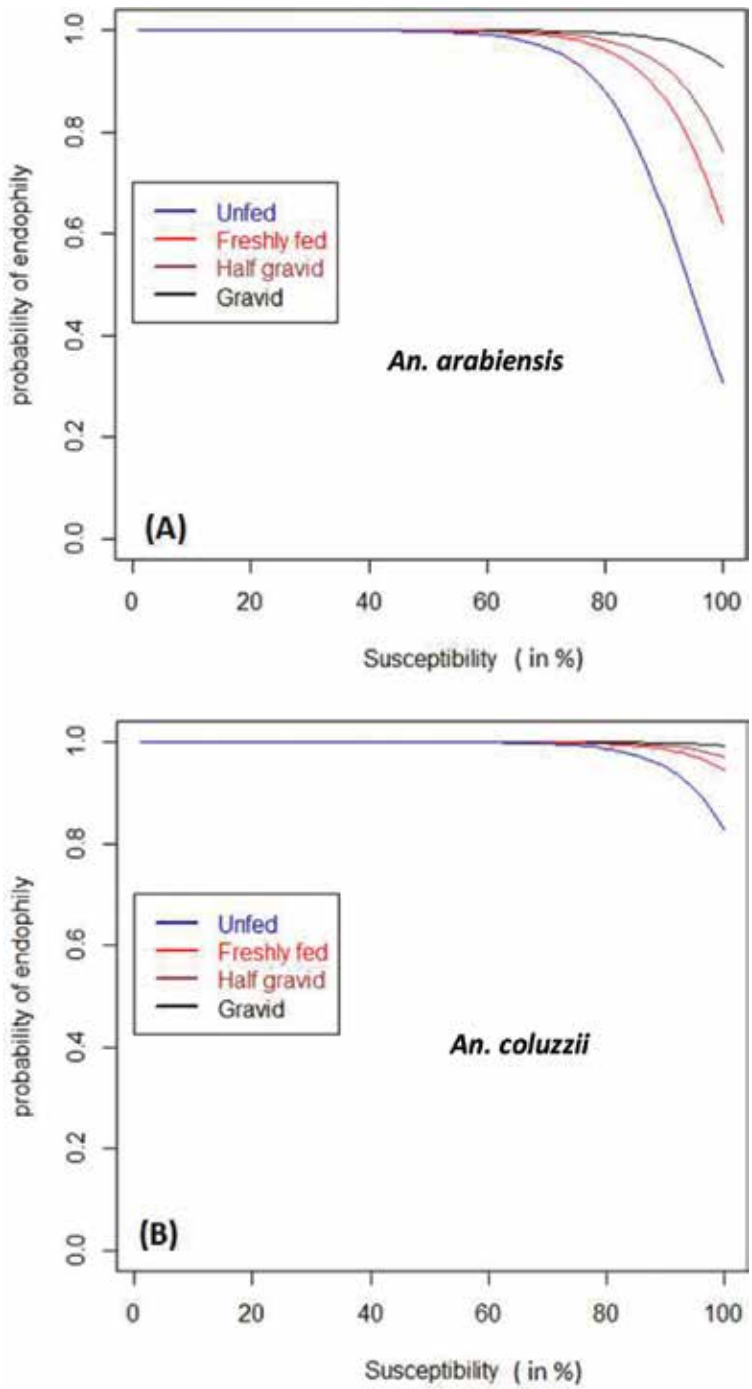

Figure 3. Endophily curves for *An. arabiensis* (A) and *An. coluzzii* (B) in relation to physiological statuses and mortality rates to susceptibility tests.

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0.62 = 0.38) and half gravid specimens (total difference in probability was 1.00 − 0.76 = 0.24). The effect of deltamethrin resistance on the resting behaviour of gravid *An. arabiensis* was not significant.

With specimens of the *An. coluzzii* species, the effect of deltamethrin resistance on the resting behaviour was not significant ($p > 0.8$) in general as shown in **Figure 3(B)**. Nevertheless, the probability of endophily in unfed *An. coluzzii* was slightly decreased between 80 and 100% mortality rates.

2.4. Discussion

The current study revealed high indoor densities and an obvious infectivity of deltamethrin-resistant *An. arabiensis* and *An. coluzzii* populations, leading to contact with humans and ongoing malaria transmission in the study districts no matter high coverage of LLINs.

Most important malaria vectors such as *An. gambiae*, *An. arabiensis* and *An. funestus* [34] prefer to feed in the middle of the night when most humans are typically asleep, immobile and vulnerable to attack. Feeding indoors at night is, therefore, the behaviour that is targeted by the use of LLINs to protect sleeping humans. Indoor feeding is then often followed by resting within the same sheltered domestic structure for one or two nights while the blood meal is digested and eggs are developed. Applications of insecticides to houses by IRS, to kill mosquitoes resting on inner surfaces of the walls and roof after they have fed upon the human occupants, are therefore a highly effective strategy for controlling populations of vectors that rest indoors as a matter of preference. Overall, the community-wide mass effect of LLINs and IRS can have a dramatic impact on the population size of stereotypical vectors that depend heavily upon feeding on humans and resting inside houses [35]. However, there are a number of possible impacts that insecticide use indoors could have on mosquito behaviour including changes in biting phenology, the rate of endophagy and endophily. Therefore, understanding that the limits of IRS, LLINs or any other vector-control strategy are primarily defined by the behavioural traits of mosquitoes [36–38] is fundamental. Patterns of malaria vector behaviours have been examined in quite a good number of studies where vectors undergo modifications on their behaviour to facilitate avoidance or circumvention of insecticides [39]. However, better understanding the interactions between insecticide resistance phenotypes in field malaria vector populations, the resistance mechanisms and subsequent behavioural patterns in the presence of LLINs at household level is essential.

The phenotype of deltamethrin resistance in *An. arabiensis* and *An. coluzzii* recorded in this study was consistent with previous studies conducted in the north Cameroon Region [11, 19, 40]. Furthermore, these data provided evidence of widespread deltamethrin resistance in species of the *An. gambiae* complex from this region. In *An. arabiensis* field populations from Pitoa, a set of constitutively overexpressed antioxidant genes (superoxide dismutases *SOD2* and *SOD3*, the glutathione S-transferase *GSTS1* and the thioredoxin-dependent peroxidase *TPX4*) and a single P450 (*CYP4G16*) were previously associated with increased tolerance to deltamethrin [15]. Indeed, metabolic-based resistance was likely the main DDT and pyrethroid resistance mechanisms in *An. gambiae s.l.* in this area, since the *kdr L1014F* allele, which is

recessive, was found at low frequency and heterozygous state in August 2011 just before the current survey [40].

The most abundant species of the *An. gambiae* complex encountered in the three study health districts was the *An. arabiensis*, while *An. coluzzii* and *An. gambiae s.s.* occurred in small ranges. The predominance of *An. arabiensis* in the analysed samples was consistent with the distribution of species of the *An. gambiae* complex in north Cameroon [11, 15, 19]. Since data collection for the current study was done between October and November 2011, the observed distribution of sibling species of *An. gambiae* complex may vary periodically [41]. Significant variations in the relative frequencies of *An. arabiensis* and *An. gambiae* were previously recorded over time and between localities in the north Cameroon. More interestingly, both species were resistant to DDT and deltamethrin, and the resistance steadily increased throughout the rainy season [11]. In other studies, *An. arabiensis* populations have shown some variations in biting or resting behaviour at different locations, with some of these variations being explained by seasons or historical use of insecticides [42].

Substantial variations have also been recorded in the current study. Most of the indoor-collected samples were freshly fed or unfed, suggesting that the vectors might tend to increase the chance of feeding on humans by remaining indoors, regardless of the presence of the LLINs. This behavioural pattern could therefore lead to increasing malaria risk in north Cameroon, since the sporozoite rates in mosquito samples were 4.8 and 1.1% for *An. coluzzii* and *An. arabiensis,* respectively, suggesting regular malaria transmission in households with LLINs. Furthermore, the human blood index was high (56–80%) for the three sibling species, demonstrating their anthropophilic tendencies. Further variations were linked to mosquito physiological status. While all the four gonotrophic states were found in analysed *An. arabiensis* samples (indoors or exiting), only unfed and freshly fed gonotrophic states were found in *An. coluzzii* and *An. gambiae s.s.* samples, suggesting outdoor-resting habit (exophily) for half gravid and gravid mosquitoes of these two species, although the small size of the collected and analysed samples ($N = 21$ for *An. coluzzii* and $N = 7$ for *An. gambiae*) precludes any strong conclusion in these cases. Around 40% of half gravid *An. arabiensis* samples were found in exit traps and almost the same proportion of specimens of this species displayed animal blood meals (cattle, pigs and sheep), sometimes mixed with human blood. This variability of resting behaviour of *An. arabiensis* and to a lesser extent *An. coluzzii* was confirmed by the two-level logit regression analysis in relation with deltamethrin resistance. The decrease of insecticide resistance tended to induce exophily in unfed specimens, while the probability for gravid *An. arabiensis* to remain indoors no matter their susceptibility status was high. Although endophily of gravid mosquitoes was consistent with blood digestion and egg development processes, it could also be enhanced in the specimens displaying resistance to deltamethrin.

Data reported in this study put forward a high plasticity of feeding and resting behaviour in the three species of the *An. gambiae* complex from the study areas. Accordingly, resistant malaria vectors, which have developed the capacity of blood feeding or resting in houses in the presence of LLINs, render LLINs useless if they are not in good state (torn, holed or not large enough). The physical integrity of LLINs in the specific context of vector resistance to

http://dx.doi.org/10.5772/65463

pyrethroid insecticides is essential for the sustainability of malaria mass prevention. These findings concord with those reported in Benin, where households in the areas with resistant mosquitoes showed high rates of blood feeding, while freshly insecticide-treated nets provided no protection once holed. By contrast, sleeping under a holed LLIN in the location where susceptible mosquitoes were common decreased the odds of being bitten by 66% and the majority of mosquitoes were killed by the treatment [43].

When the LLIN is intact, a side effect of physiological resistance would be the reduction in vector behavioural responsiveness to the insecticide [44–46]. Pyrethroid-resistant mosquitoes showed reduced irritability to insecticides, which allow them to rest on the surface for longer periods than susceptible mosquitoes, thus increasing the dose of insecticide received and likely the subsequent mortality rates [46]. In most cases, the effect of physiological resistance is unquantified and dependent on the mechanism of resistance [45]. The *Kdr* mutation is considered a relatively weak form of resistance compared to metabolic-based resistance, and it is usually only when the *kdr* occurs along with metabolic-based resistance that control fails [47, 48]. Furthermore, the current study has demonstrated that control failure may also result from a cumulative effect of insecticide resistance mechanisms and the profile of physiological status of targeted vector population.

With untreated nets, such effects of insecticide resistance and behavioural traits may not occur, and personal protection may be afforded provided the nets are tucked in, maintained in good condition and sufficiently large so that the sleepers do not make contact with the net [49]. Subsequently, this protection may result in a reduction of malaria morbidity compared with areas without nets at all. However, the obtained level of protection remains lower than that conferred by the LLINs which remain one of the most cost-effective tools for malaria mass prevention, even in some areas where vectors have developed pyrethroid resistance [46, 50, 51].

According to WHO, when insecticide resistance is confirmed, pre-emptive actions must be taken to manage this resistance and to ensure that the effectiveness of insecticides used for malaria vector control is preserved [25]. Trials of the combination of LLINs and IRS with different classes of insecticides (carbamates or organophosphates) as a resistance management strategy are necessary in north Cameroon, in order to guide malaria vector-control strategies in this region.

3. Conclusions

The data generated in the current study provide further evidence for cumulative effects of deltamethrin resistance and physiological status of *An. arabiensis* and to lesser extent *An. coluzzii* populations on the patterns of their behavioural responses to LLINs in north Cameroon. It is unique in drawing a model based on analysis of insecticide resistance, indoors/exiting mosquito densities and physiological statuses data. The high densities of vectors inside houses and regular contact with human may lead to increasing malaria transmission in study health districts regardless of the coverage of LLNs. These findings are valuable for the development of resistance management strategies, and effective tools for malaria control and elimination in the study areas.

Acknowledgements

This study was funded by the Bill and Melinda Gates Foundation (Grant Number 4849901) and the World Health Organization. We wish to thank the communities of the three study health districts in north Cameroon for their active participation in the survey and collaboration with the research team.

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Chapter 20

Secondary Malaria Vectors of Sub-Saharan Africa: Threat to Malaria Elimination on the Continent?

Yaw Asare Afrane, Mariangela Bonizzoni and Guiyun Yan

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65359

Abstract

Secondary vectors of malaria include those anopheline species that are known to play minor part in malaria transmission. Primary vectors of malaria in Africa are *Anopheles gambiae* s.s, *Anopheles coluzzii*, *Anopheles arabiensis*, *Anopheles funestus*, *Anopheles moucheti* and *Anopheles nili*, while *Anopheles rivolorum*, *Anopheles pharoensis*, *Anopheles ziemanni*, among others are secondary vectors. They are recognized for their importance in malaria transmission, as they may help to augment or extend the malaria transmission period and potentially sustain malaria transmission after the main indoor resting and indoor biting vectors have been reduced by vector control measures such as indoor residual spraying or Long-lasting insecticidal nets (LLINs). Thus, the terminology "secondary" versus "primary" vector is fluid and forged by ecological conditions and malaria control strategies. Most secondary vectors are outdoor resting and outdoor biting are thus, not taken care of in the current control methods. High use of insecticides for vector control in Africa, climate change, unprecedented land use changes in Africa are some of the factors that could influence the conversion of secondary vectors to become main vectors in Africa. This chapter examines the role of secondary vectors in malaria transmission and the possibility of them becoming main vectors in future.

Keywords: secondary vectors, main vectors, exophilic, exophagic, malaria elimination, residual malaria transmission

1. Introduction

Malaria is still a major public health problem in sub-Saharan Africa despite the massive investment in intervention measures that have been rolled out within the last decade and have

produced a decline of 37% of malaria cases [1]. Main interventions include the scaling up of vector control through long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [2], as well as the introduction of ACT and improved malaria diagnostic [1]. Malaria transmission dynamics within sub-Saharan African countries is highly variable. Transmission can occur throughout the year (in particular with areas that receive rainfall twice a year) or only during a few months in the year (in particular with areas that has only one transmission season) and even then heterogeneities in transmission are observed between years within the same area. Inoculation rates vary from 0 to over a 1000 infective bites per year. Some areas have sole vectors that are involved in transmission of parasites to the human population while others could have several vectors that will consist of main vectors and secondary vectors. Differences in ecological requirements, longevity and feeding behavior (e.g. anthropophily and endophily) account for the different roles played by main and secondary vectors in malaria transmission in Africa [3].

Secondary vectors of malaria include those anopheline species that are known to play or suspected of playing a minor part in malaria transmission. With over 140 species of anopheline species in sub-Saharan Africa, <20 of them are able to transmit malaria to humans [4]. There are some six species that are considered to be major malaria vectors that are responsible for 95% of the total malaria transmission on the continent [5]. These are *Anopheles gambiae* Giles, *Anopheles coluzzii* Coetzee & Wilkerson sp. n, *Anopheles arabiensis* Patton, *Anopheles funestus* Giles, *Anopheles moucheti* Evans and *Anopheles nili* Theobald [5, 6]. The remaining (5%) is transmitted by "secondary vectors" or "vectors that are normally of local importance" [5] that include *Anopheles rivolorum* Leeson [7], *Anopheles pharoensis* Theobald [8], *Anopheles coustani* Laveran [9], Anopheles *ziemanni* Grtünberg [10] *Anopheles squamosus* Theobald [11]. Secondary vectors have been recognized for their importance in malaria transmission, as they may help to augment or extend the malaria transmission period [12, 13]. Moreover, it is known that many of these secondary vectors are exophilic (outdoor resting) and exophagic (outdoor biting) and therefore has the potential to sustain transmission of malaria after the main vectors have been reduced by indoor control measures such as indoor residual spraying or insecticide-treated bednet (ITN) use [3, 8, 14].

The current malaria vector intervention tools are all indoor and insecticide based. This is because the main malaria vectors in Africa are majorly endophagic and endophilic. Secondary vectors that could be exophagic and exophilic or could bite earlier indoors before people sleep under their LLINs and those that move between being zoophilic (tendency to bite animals only) and anthropophilic (tendency to bite humans only) are left out of the current control methods. However, a few secondary vectors have been known historically to flourish and take over malaria transmission after the main vector(s) have been suppressed [7]. High use of insecticides for vector control in Africa, climate change, unprecedented land use and land cover changes that is ongoing in many parts of Africa are some of the factors that could influence the conversion of secondary vectors to become main vectors in sub-Saharan Africa.

Secondary vectors are species that frequently have relatively little contact with man and are perhaps less likely to be affected by house-spraying with residual insecticides and the use of insecticide impregnated bednets than are the primary vectors. This chapter emphasizes the

http://dx.doi.org/10.5772/65359

importance of such secondary vectors. It examines the role of secondary vectors in malaria transmission and the possibility of them becoming main vectors in future, as many countries in sub-Saharan Africa drive towards the elimination of the disease. It highlights the overall malaria parasite transmission intensity by these secondary vectors in several sites across Africa. Historical evidence is presented in this chapter to underscore the possibility of secondary vectors becoming main vectors. For instance, *An. arabiensis* was considered a secondary vector of malaria some decades back but is now one of the most important vectors in Africa.

2. Contribution of secondary vectors to malaria transmission in different parts of sub-Saharan Africa

Secondary vectors just like the main vectors are distributed all over sub-Saharan Africa. From Senegal in the west of Africa through Cameroun to Ethiopia and down to Angola, each country has a few of them that could be either transmitting malaria or not and all of them contributes to 5% of malaria transmission on the continent. They are therefore of importance to the sustenance of malaria transmission. Some secondary vectors have historically been known to be transmitting sporozoites of malaria parasites, albeit, at a lower rate whilst some vectors have been known to bite man but have not been found carrying malaria parasites. For instance, the *An. coustani* have been reported to be carrying sporozoites of malaria parasites in Tanzania [8] and the Democratic Republic of Congo [10] in whilst *An. ziemanni* were found in Ethiopia and Cameroon in the 1950s to be infected with *Plasmodium* sporozoites [10]. *An. rivolorum* was also found in Kenya and Tanzania transmitting malaria in the late 1950s [7]. *An. pharoensis* was found with sporozoites in Tanzania [8] and Baukina Faso (formerly Upper Volta) [15]. *An. squamosus* sensu stricto Theobald was found with sporozoites in Muheza, Tanzania [11]. The following is an account of these secondary vectors and their contribution to malaria transmission according to the sub-regions of sub-Saharan Africa.

2.1. Western Africa

Around the Senegal River delta, *An. pharoensis* Theobald has been found to be the most prevalent man-biting anopheline mosquito. In one area, 5/912 of *An. pharoensis* examined were sporozoite positive [16], while in another area 3/396 were infected with *Plasmodium falciparum* sporozoites [17]. These were all living in sympatry with the main vector *An. gambiae* Giles sensu lato of which 98 were caught and none were positive for malaria sporozoites. Only 1/3076 of *An. funestus* was found to be with sporozoites. Other secondary vectors found there included, *An. coustani*, *Anopheles wellcomei* and *Anopheles rufipes*. These studies suggest that *An. pharoensis* has a bigger role to play in malaria transmission in this area of Senegal, than even the main malaria vectors.

In Gambia, presumed secondary vectors are *An. pharoensis, An. ziemanni, An. squamosus* and *An. rufipes,* which were caught in experimental huts. Their role in malaria transmission is still uncertain as Plasmodium infections were not checked [12, 13]. In Côte-d'Ivoire, *An. ziemanni*

was the most abundant species (32.5% of the total vectors) caught as larvae in rice paddies in the western part of the country [14].

In Ghana, *An. pharoensis, An. coustani* and *An. rufipes* were the main secondary vectors that have been sampled. In the coastal savannah area of Kpone-on-Sea, *An. pharoensis* was sampled among other main malaria vectors using human landing catches (0.1 of 1233 vectors caught) [18]. *An. coustani* was also sampled (6/1642) in a nearby urban Accra also using human landing catches. In the forest-savannah transitional town of Kintampo, *An. rufipes* was sampled in night catches using CDC light traps and constituted 370/9391 of anophelines that were captured [19]. None of these vectors were tested by the authors for the presence of malaria sporozoites, and therefore, their contribution to malaria transmission in Ghana is not known; however, the fact that they were caught in human landing catches suggest their strong anthropophilic tendency.

In Baukina Faso, 3/3385 of *An. ziemanni* and *An. coustani* collected haboured sporozoites of malaria parasites [15]. In Benin, main secondary vectors were *An. pharoensis* and *An. coustani* in night catches in the northeastern Benin [20] whilst *An. ziemanni*, *An. pharoensis* and *An. coustani* have been sampled in southeastern Benin [21]. However, none were infected with Plasmodium parasites.

2.2. Central Africa

In irrigated rice fields in Goulmoun in south western Chad, four vectors were identified with a different human biting rate: *An. arabiensis* at 51 bites/human/night was the most efficient biting vector, followed by *An. pharoensis* (12.5), *An. funestus* (0.15 b/h/n) and *An. ziemanni* (1.3 b/h/n). The circumsporozoite protein rate was 1.4% for *An. arabiensis*, 1.4% for *An. funestus*, 0.8% for *An. pharoensis* and 0.5% for *An. ziemanni*. The overall annual EIR was estimated at 311 bites of infected anophelines/human/year, contributed mostly by *An. arabiensis* (84.5%) and *An. pharoensis* (12.2%) [22]. This study revealed the implication of *An. pharoensis* and to some extent *An. ziemanni* in malaria transmission in the area complementing the major role played by *An. arabiensis*.

Cameroun seems to be the country with most secondary vectors implicated in malaria transmission. In northwest of Cameroon, *An. ziemanni* was the main malaria vector. It was found in both outdoor and indoor catches with a range of 6.75–8.29 b/p/n and 0.063 infectious bites per person per night (ib/p/n) [23]. Studies have shown nine secondary vectors with history of carrying *Plasmodium sporozoites, namely*: An. *coustani* Laveran, *Anopheles ovengensis* Awono-Ambene et al., *Anopheles carnevalei* Brunhes et al., *Anopheles hancocki* Edwards, *Anopheles marshallii* (Theobald), *Anopheles paludis* Theobald, *An. pharoensis* Theobald, *An. wellcomei* Theobald and *An. ziemanni* Grtünberg [3, 12]. These constituted 11% of all anophelines sampled and with infection rate of 1.36% compared to 3.08% for the main vectors that live in sympatry with them. *An. pharoensis* and *An. ovengensis* were repeatedly found infected by *P. falciparum* and contributed substantially to the total malaria transmission intensity in some areas where they were abundant. Though these vectors showed strong exophilic and/or exophagic habits, they might elude vector control directed against endophilic and endophagic malaria vectors. In this same area, *An. pharoensis* has been reported since 1961 to be carrying Plasmodium

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infections [23, 24]. This shows that when conditions become favorable or when it becomes the most abundant vector in the area, it has the potential to assume the role of the main vector.

Anopheles coustani complex mosquitoes have been found harboring sporozoites in Katanga in the Democratic Republic of the Congo [10]. *An. paludis* Theobald, a member of the *An. coustani* group, has been reported as a vector in the Congo with sporozoite rates as high as 10% [25].

2.3. Eastern Africa

Anopheles pharoensis and *An. ziemanni* seems to be the most important secondary vectors in east Africa. In Ethiopia, *An. pharoensis* is the most prevalent secondary vector. It was the second most abundant malaria vector after *An. arabiensis* in irrigated rice paddies in central part of the country. *An. pharoensis* showed a slight preference for human blood (63.6%) over bovines (49.5%) and had 7.5 infective bites per person per year compared to 27.3 for *An. arabiensis. An. coustani* was also sampled in this study but none of them was infected with any Plasmodium parasites [26]. In an area close by in south-central Ethiopia, *An. pharoensis* was sampled with high human blood index (HBI) and carrying *Plasmodium vivax* [27]. *An. pharoensis* and *An. ziemanni* were also sampled in another study, most of which had taken human blood meals but none infected with Plasmodium parasites [28].

In Kenya, *An. pharoensis* was sampled from the Mwea irrigation scheme in Central Kenya, and it constituted 15.69% of the total anopheline catches, with *P. falciparum* sporozoite rates of 1.3% by ELISA and 0.68% by dissection, while those for *An. funestus* were 1.7% by ELISA and 1.25% by dissection [13]. In the same area, *Anopheles parensis* was the main member of the *An. funestus* species group found resting inside human dwellings in Mwea area of central Kenya. Even though none of them were positive for *P. falciparum* sporozoites, they had high human blood index (HBI) indicating that they have been biting humans indoors [29].

Anopheles ziemanni Grunberg was sampled resting inside several human dwellings in western Kenya. Although none of the *An. ziemanni* sampled were infected with *P. falciparum* sporozoites, the density of this species and their human blood index compared well with the other main vectors. This suggest that there is a possible role in malaria transmission [30]. Another study [31] also found *An. ziemanni* to have an HBI that was not significantly different from that of *An. arabiensis*, an important vector of malaria in Kenya and especially in rice irrigation schemes. Studies carried out in Ethiopia and Cameroon in the 1950s each found *An. ziemanni* mosquitoes to be infected with *Plasmodium* sporozoites [32], this suggests that this mosquito species is indeed susceptible to malaria parasites and can play a role in malaria transmission. Earlier studies in the same area also found proportions of *An. ziemanni* out of all mosquitoes collected that were higher than those of *An. gambiae s.l.* and *An. funestus* by factors of 2.6 and 43.7, respectively [33].

In Tanzania, *An. squamosus, An. coustani and An. ziemanni* have been historically implicated in malaria transmission [9].

2.4. Southern Africa

Anopheles coustani s.l. Laveran and *An. squamosus* Theobald have been reported in southern Zambia to demonstrate an unexpected high degree of anthropophilic tendencies, even though they have generally been of negligible importance to malaria transmission due to their overwhelmingly zoophilic behavior. They also have been found foraging during early evening and the majority of blood meals from these mosquito species were from human hosts. Although no *An. coustani s.l.* or *An. squamosus* were found to be positive for Plasmodium species, the demonstrated anthropophilic tendencies of these mosquitoes in southern Zambia suggest their potential as secondary vectors of malaria [34]. In eastern Zambia, Lobo et al. [9] also observed unexpected number of sporozoite positive mosquitoes in some secondary vectors, namely, *Anopheles rivulorum* (2/30), *Anopheles theileri* (2/14) and the *An. coustani* group (12/340). *An. coustani* was more anthropophilic than its siblings.

Similarly, studies from Mozambique displayed high anthropophilic behavior with early peaks in foraging activity with *An. coustani s.l.* There was a combination of outdoor and early evening foraging behavior for these species and this could increase their potential as secondary vectors in areas where indoor control measures such as indoor residual spraying or LLINs are employed [35].

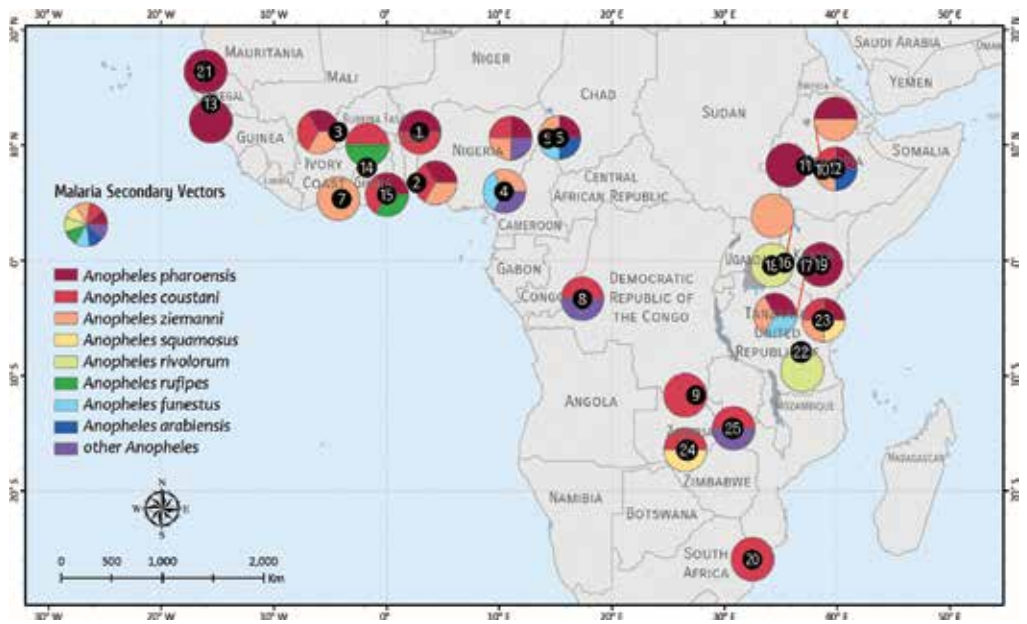

Figure 1. A map showing the distribution of the secondary vectors discussed from sub-Saharan Africa.

All these shows that these secondary vectors do assume anthropophilic behaviors, could get infected with plasmodium infection and could potentially become the main vector of malaria. **Figure 1** shows a map of the distribution of all the secondary vectors discussed here in this review from sub-Saharan Africa.

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3. Impact of land use and land cover changes on mosquito species proliferation

Environmental change is driving the expansion of numerous vector species and the intensification of pathogen transmission in many places in the world [36] because vectors respond sharply to changes in the ecology of their breeding habitat. These ecological changes include land use and land cover changes [37–40] which can change the environment within which the vector prefers to breed or the microclimate of the area within which certain vectors would tolerate [40]. Malaria vectors could therefore invade a new area when the land use and land cover changes [41]. Land cover changes and human settlement subsequent to deforestation has prompted an increase in the human-biting rate of formerly zoophilic vectors in several parts of the tropics and the instigation of upsurges in malaria transmission and malaria epidemics [40, 42]. Human settlement can increase malaria transmission if there are malaria infected people in among the settlers. Mitigating against the impacts of environmental change on malaria transmission will be particularly difficult when public health goals conflict with economic development. Economic development in many places in Africa is associated with extension of agricultural practices such as rice and sugar cane that are associated with extensive water bodies that favor the establishment of breeding sites for malaria vectors. Economic development has also been associated with deforestation, where the forest is cut down for housing and agricultural purposes. For instance in Guiana, following the elimination of malaria in the Demerara River Estuary by DDT spraying, the human population grew rapidly and land use activities switched from livestock herding to more profitable rice farming. This caused the formerly zoophilic *Anopheles aquasalis* to switch from being zoophilic to an anthrophilic behavior. This change initiated the return of transmission into the area after 16 years of absence [43]. Again in Swaziland, resurgence of malaria cases after elimination in 1959 was due to agricultural developments during that time that involved irrigation projects for sugar cane cultivation, which created conditions conducive for malaria vectors to breed and flourish. Vector density increased, with subsequent increase in biting frequency, as no animals were around the area to serve as alternative hosts. The resurgence of malaria was also influenced by migrant workers who came to the area from disease-endemic areas of Mozambique, some of whom were parasite-carriers, in the 1960s and early 1970s [44].

In many areas of Africa, the type of land use activity and the ecological context created after deforestation, determines which species of mosquito are able to remain and adapt, which ones disappear, and which new species are able to invade the place, that find the new habitat congenial to their survival and proliferation [45]. Deforestation could enhance the vectorial capacity of malaria transmitting mosquitoes, and there was 29–106% increase in vectorial capacity for *An. gambiae* in deforested areas compared with forested areas in western Kenya [46]. In the same area, it was found that deforestation increases water temperature of larval habitat, hence increase larvae survival, population density and gametocytes development in adult mosquitoes [38, 46, 47]. In western Africa, deforestation and irrigation have been followed by an increase in *P. falciparum* malaria transmitted by *An. gambiae* in villages close to forest, *An. funestus* in the savannah, and *An. arabiensis* in urban and peri-urban areas [48]. In

northern Cameroon, changes to the ecology of the area along the Bamendjim dam has resulted in high densities of *An. ziemanni*, a secondary vector that is now playing the role of a primary vector [49]. The changes to the ecological settings have enabled the proliferation of breeding sites contributing to its development.

Environmental pressures and climate change may bring about malaria vectors dynamism, which leads to some malaria vectors becoming more efficient in transmitting malaria [50]. Manga et al. [41], working in an area that has been deforested to build a new airport in Cameroon, observed that deforestation caused the introduction of *An. gambiae* into a habitat that was previously predominated by *An. moucheti*. Changes to the forest cover led to the subsequent replacement of the vectors in the area, which could worsen the malaria situation in the area.

Outside of the continent of Africa, Conn *et al.* [42] found in Amapa state, Brazil that *Anopheles marajoara*, has become the main malaria vector species in this area, when it previously was of minor importance. This species occurred in much greater abundance compared with the presumed vector *Anopheles darlingi*. Also, a significantly higher proportion of *An. marajoara* was infected with malaria parasites. This was attributed to increased alterations in land use, invasion of its primary breeding sites by human immigrants, and its anthropophilic behavior. This finding highlights a challenge in malaria control, that the targeting of specific vectors may be complicated by a changing mosaic of different locally important vectors and their interactions with human populations.

4. Proliferation of mosquito species to higher altitudes due to climate change and climate variability

The highlands of Africa, where malaria incidence is on the rise, represent an ecological zone of special concern [51]. The high rate of deforestation leads to rise in temperatures in highland areas [39, 52, 53]. Global climate warming could potentially make the high-altitude areas which used to be unsuitable for mosquito proliferation suitable for these mosquitoes to increase in density. Each vector has its own ecological niche requirement, and an important limiting factor for vector spatial distribution range is climate. A typical case in point, in the highlands of western Kenya, Zhou et al. [54] reported that the population of *An. arabiensis* rose from >1% in 2003 to 18.8% in 2009. Again, *An. arabiensis* have been absent in the highlands of central Kenya, however, studies by Chen et al. [55] reported the presence of *An. arabiensis* in these highlands which have elevation of 1720–1921 m above sea level for the first time. This suggests that the ecological conditions or local climate have become conducive to the proliferation of this vector species.

In the Amani hills of Tanzania, Matola et al. [56] reported that malaria vectors were scarce on the Amani hills until the late 1960s, and it was generally presumed that any cases of malaria transmission must have been contracted by people visiting surrounding lower altitudes where malaria is holoendemic. However, *An. funestus* and *An. gambiae* both became more abundant during the 1970s and 1980s with high sporozoite-positive specimens of both. Malaria asexual

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parasite rates increased dramatically in the early 1908s, with the percentage of children below 1 year who had parasitamia and whose parents reported not visiting lowland localities away from the Amani hills increased drastically, suggesting possible local malaria infection. The conclusion was that various factors including climatological changes, and increased deforestation for agricultural activities have contributed to this changed malaria endemicity and transmission.

Even though these vectors are main and not secondary vectors, the fact that the highlands became permissible to their proliferation leading to increases in malaria transmission suggest that this could happen with secondary vectors. Some of these secondary vectors already live in the highland areas [30, 57] and therefore when conditions such as land use changes, climate change and reduction in interspecific competition from main vectors as a result of elimination or reduction in their population could trigger their proliferation. Others could migrate into the highlands from surrounding lowland areas when conditions such as those already discussed become permissible to their survival. Malaria vectors and non-vectors could periodically extend their range beyond their normal area of distribution.

5. Impact of intensive use of insecticides for public health interventions

Insecticides are the primary weapon against malaria vectors in the current malaria intervention paradigm. However, their prolonged use have been associated with the development of resistance by malaria vectors. Their intensive application has evolutionary implications evident in the number, behavior and physiology of the vectors. For numeric responses to intensive insecticide use, mosquito populations typically decrease in density because their longevity is reduced very much [58, 59]. For instance, studies carried out in western Kenya showed that, *An. gambiae s.l.* and *An. funestus* population density declined markedly in treatment compared to control villages in a randomized trial of insecticide treated bed nets [60]. This effect persisted for 3 years after the trial ended [61]. For behavioral responses, females of some *Anopheline* vectors showed elevated activity due to excitation effects of the active ingredients in insecticide formulations of insecticide-treated bednets or indoor residual sprays, which resulted in their movement away from the insecticide source, irrespective of having obtained a human blood meal [62, 63]. Evolutionary responses typically involve changes in phenotypic sensitivity to the insecticides being used, when alleles associated with reduced target site sensitivity or enhanced metabolic detoxification increase in frequency [64].

The reduction in one target vector may trigger a cascade of ecological effects that could impede or enhance transmission by another. A notable examples include the apparent replacement of the highly anthropophilic and endophagic *An. funestus* by the less potent vectors *Anopheles rivolurum* Leeson and *An. parensis* in Kenya and Tanzania following house spraying campaigns in the 1950s [7]. *An. rivolurum* population rose up to about seven times its former density. During this period also, *An. gambiae s.s.* also declined markedly in their population. Quiet recently, there have also been reported increase in population size of *An. rivulorum* Leeson, in western Kenya lowland areas where there have been high coverage of LLIN distribution. This

vector has shown early outdoor biting activity [65]. These changes could be attributed to a reduction in interspecific competition caused by the intervention that allowed these secondary vectors to move into the niche formerly occupied by *An. funestus*. In regions with sympatric vector species where insecticide-based vector control are used, malaria vectors that do not enter houses will have selective survival advantage over vectors that do enter houses, due to the latter's exposure to the insecticides used indoors. This will result in a selective decline in the density of house-entering endophagic vectors relative to more exophagic species. In such situations, exophagic vectors in the area which may be considered secondary in importance could maintain transmission [66].

Again, in Kenya, the inception of rigorous malaria control in the early part of the 2000s using LLINs saw dramatic changes of vector species. There has been a marked decline in the population of *An. gambiae s.s.* and an increase in the population of *An. arabiensis* as household ownership of bed nets rose over a 10-year period [54, 67]. Similarly, in Tanzania, there are shifts in species composition due to the use of ITNs, which resulted in a more dramatic drop in the density of highly anthropophagic and endophagic *An. gambiae s.s.* relative to the zoophagic and adaptable *An. Arabiensis* [68]. Most recently, there are reported new species that have emerged in western Kenya [69]. These species of mosquitoes did not match the morphologic descriptions of any of the more recently identified species. This demonstrates the presence of outdoor-active, early-biting potential malaria parasite vectors not previously described in Kenya. It has not yet been proven whether these vectors existed or are entirely new species. Their overall biology needs to be studied to understand their role in malaria transmission.

These scenarios demonstrate that secondary vectors have the potential to occupy the niche left by main vectors after the latter's elimination through intensive vector control in sub-Saharan Africa that relies solely on insecticides with the use of IRS and LLINs.

6. Commentary

Malaria transmission in Africa is a dynamic and complex system that is continuously changing. Despite the substantial amount of work done on malaria epidemiology and control in Africa, there remains gaps in our understanding of the ecology and biology of secondary vectors. Further knowledge is required to improve control of the disease especially as many countries embark on rigorous campaign to move from control to elimination phase of malaria transmission. Currently, much attention has been given to the main malaria vectors with the promotion of high LLIN use and IRS application which mainly tackle indoor transmitting vectors. However, a very big public health problem in recent years is residual malaria transmission. This has been reported to be increasing in many parts of sub-Saharan Africa [68, 70]. Most often studies on residual malaria transmission tend to focus on the main malaria vectors. However, the secondary vectors discussed here are mostly exophagic and exophilic and therefore would be more involved in residual transmission. The contribution of these secondary vectors should be seen much more in influencing residual malaria transmission. Moreso when there is currently no intervention in the vector control paradigm to take care of residual

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malaria transmitting vectors. Other measures such as larviciding or larval source reduction that could tackle secondary vectors and residual malaria transmission have not receive much attention. There is a great need to understand the bioecology of secondary vectors and their contribution to malaria epidemiology in order to program intervention for them.

From the above review, it was seen that *An. pharoensis*, *An. coustani* and *An. ziemanni* in particular are secondary vectors that are prevalent in almost every part of sub-Saharan Africa, right from Senegal in western Africa to Ethiopia in the east and down to Mozambique and Angola. They have shown in their ecology that, they could be anthropophilic since they were caught in human landing catches and also carried *P. falciparum* sporozoites as well as *P. vivax* in Ethiopia. Their exophilic behavior means they have the potential to increase residual malaria transmission wherever, they are found. The fact that in some instances they have been found in indoor collections [29, 49] shows that they could also become endophagic and endophilic and have the potential to occupy a new niche if the main vectors are eliminated or their population becomes suppressed. The ecology and population dynamics of these secondary vectors should be monitored as many countries in Africa move towards the elimination phase of malaria epidemiology.

Why the population densities of these secondary vectors have not been as much as the main vectors has received little attention in the research world. It could be that interspecific competition with the main vectors has not favored the secondary vectors. If this is true, then when main vectors are eliminated or their densities brought down by intervention, secondary vectors could assume the role of main vectors since there would not be any competition. However, Gillies [71] asserted that most secondary vectors do have a short lifespan with natural mortalities estimated to be around 50–60% per gonotrophic cycle. This could explain to an extent, why their population sizes have not been high in many places. However, it has been shown in several areas in sub-Saharan Africa that the rigorous LLIN distribution and IRS application for malaria control within the last decade has led to *An. arabiensis* becoming the main vector when the population of *An. gambiae s.s*. was suppressed by the high insecticide use for vector control. Also, in the late 1950s, *An. rivolorum* replaced *An. funestus* in east Africa after 18 months of indoor residual spraying with dieldrin [7]. Also in the late 1950s, *An. darlingi* which was the primary vector of malaria was eliminated from Venezuela, but malaria of low endemicity due to the secondary vectors *Anopheles albimanus* and *Anopheles nuneztovari* still persisted for many years in certain areas [66]. These show that it is possible for secondary vectors to become the main vectors as many countries in Africa move towards elimination of malaria through vector control.

Human behavior is identified to drive residual malaria transmission. In areas where it is warmer in some months of the year, some residents would want to sit outside of their house instead of being indoors for several hours of the night or sleep outside the whole night as happens in the north of Ghana [72]. In such areas, if the main vectors are eliminated, it is more likely that secondary vectors would replace them since blood meal will be available outdoors and possible pathogen transmission would be enhanced. It has also been suggested that since many secondary vectors are exophagic and exophilic, they could potentially sustain transmis-

sion of malaria after the main endophagic and endophilic vectors have been reduced by indoor control measures such as IRS and LLIN use [3, 8, 14].

However, it is worth noting that a possible reason why these vectors have not been able to actively transmit malaria might be that these secondary vectors may not be as refractory to the development of Plasmodium parasites as the main vectors are. If this is true, then no matter how abundant their population might be, they may not be able to assume the responsibility as main vectors and actively increase malaria transmission.

It is worth to note that the co-occurrence of primary and secondary vectors at the same sites may lead to an increased risk of malaria transmission. High infection rates in the secondary vectors could also arise as a result of high malaria transmission maintained by the primary vectors and increased density of humans who maybe carrying gametocytes [73].

The implementation of any successful vector-control measures requires knowledge of the biology of the anopheline species present in the area to be targeted. The scientific world needs to be concerned with the bionomics, morphology and genetics of these secondary vectors, to be ready when they also step up their game to become main vectors. In addition, malaria control measures needs to take into account secondary vectors most of whom are exophagic and exophilic.

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Edited by Alfonso J. Rodriguez-Morales

Malaria is still the main vector-borne parasitic disease in the world. Fortunately, elimination of this disease was achieved in multiple countries during the last decades. During the last decade, a significant reduction of malaria in the Americas was achieved. Nevertheless, many challenges still are ahead in order to reach a higher control and to continue in the elimination toward a world free of malaria in the next decades. This book tries to update the significant epidemiological and clinical research in many aspects with a multinational perspective. This book with 20 chapters is organized into 5 major sections: (I) Clinical and Epidemiological Aspects, (II) Basic Science, (III) Therapeutics and Antimalarials, (IV) Vaccines, and (V) Entomology and Vector Control.

ISBN 978-953-51-4144-0

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